Genetic Diversity, Phylogeny and Conservation of Rainbowfish (Melanotaeniidae) in West Papua Indonesia and Its Prospect for New Ornamental Fish Commodity

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Soutenue le 03 /11 /2015
devant le jury composé de

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Odang CARMAN, Associate Professeur, IPB	(Directeur de Thèse IPB)
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Membre invite Laurent POUYAUD, Charge de Recherche IRD	(Co-directueur de thèse UM)

UNIVERSITÉ MONTPELLIER

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3. Media Fitri Isma Nugraha, Laurent Pouyaud, Odang Carman, Utut Widyastuti, M. Zairin Junior, Kadarusman, Jean-Christophe Avarre. 2015. Genetic diversity of Boeseman's rainbowfish *Melanotaenia boesemani* reared in Indonesian farms compared to endangered natural population. *Tropical Conservation Science*.Vol. 8(3): 796-805.

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1. GENERAL INTRODUCTION

The ornamental fish hobby has continuously grown over the last decades. Some endemic fish from Indonesia have been very successfully sold abroad, like arowana (genus *Scleropages*) from Borneo island, botia (*Chromobotia macracanthus*) from Borneo and Sumatera islands, Tiger barb (*Puntigrus tetrazona*) from Sumatera island, and rainbowfish (genus *Melanotaenia*) from Papua. Talking about rainbowfish (*Melanotaeniidae*), at the moment there are only 3 species cultivated in Indonesia, namely *Glossolepis incisus* Weber, 1908, *Iriatherina werneri* Meinken 1974, and *Melanotaenia boesemani* Allen & Cross, 1980. Gerald Allen, an ichtiologist from Australia, was the first to perform deep researches on rainbowfish. In addition to Gerald Allen, an Indonesian research team from the Ministry of Maritime Affairs and Fisheries (Sorong Fisheries Academy and Research and Development Center for Ornamental Fish Culture), in collaboration with French researchers (Institut De Recherche pour le Dévelopement - IRD), has conducted researches and species descriptions of this family through several expeditions conducted since 2007.

The first species of rainbowfish was scientifically described in 1843 from a collection of freshwater fishes collected in *King River* (Northen Australia) and was named *Atherina nigrans*. In 1862, Thomas Gill proposed to place this species into a new genus called *Melanotaenia; Melanotaenia nigrans* then became the first member (i.e. nominal species) of this genus. The subfamily *Melanotaeniidae* was subsequently created by Gill in 1894.More recently, Munro (1964) claimed that *Melanotaeniidae* must represent an independant family. Between 1843 and 1964, there were about 30 species described as belonging to this family. Over the last four decades, many expeditions were conducted in New Guinea, and led to the discovery of an impressive level of rainbowfish diversity. Today, this family includes 102 species in 7 genera following Nugraha *et al*., (2015) and Allen *et al*., (2015a, b): *Cairnsichthys* (1 species), *Chilatherina* (11 species), *Glossolepis* (9 species), *Iriatherina* (1 species), *Melanotaenia* (78 species), *Pelangia* (1 species) and *Rhadinocentrus* (1 species) (Eschmeyer 2014; Allen *et al.,* 2014a.b). The greatest diversity of *Melanotaeniidae* is found on the island of New Guinea with 87 endemic

species, while there are 13 species in Australia, and only 2 species spread between the two biogeographic regions (Allen & Hadiaty 2013).

Melanotaeniidae spread out throughout the island of New Guinea, as well as the large islands around the western part of the region, including Raja Ampat, Aru and Yapen islands. This fish can also be found throughout Northern Australia, along the east to the south coast of Murray Darling drainage, and in the Western part of Australia (Allen, 1991). *Melanotaeniidae* belong to the most popular freshwater fish from the island of New Guinea and northern Australia (Eschmeyer, 2014). They represent the most speciose group of pure freshwater fishes within the Australia-New Guinea region. They typically possess a compressed body covered by relatively large scales, two separate dorsal fins (the first with 3-7 spines and the second with a single spine and 6-22 segmented rays), a long-based anal fin, and no lateral line (Allen *et al.*, 2008). The family is characterized by relatively small (usually less than 10 cm) and often brightly colored fish. Sexual dimorphism is often apparent and males tend to be deeper-bodied and more vividly colored than females (Allen, 1991). Generally, they are locally abundant and occupy the full spectrum of freshwater habitats including arid-zone waterholes, swamps, rainforest streams, karst rocks and oxbow mountainous lakes and streams in the mountains.

The Boesman's rainbowfish *Melanotaenia boesemani* Allen and Cross (1980) is certainly the most popular species of rainbowfish both in Indonesia and abroad (Figure 1). This species is very different from most other rainbowfish because it harbors different colors between the anterior and posterior. The color of anterior (front) is bright blue and the color of posterior (back) is bright yellow. This species was first described by Allen and Cross (1980), from specimens caught by Marinus Boeseman in 1954-1955, which were kept in the museum *Rijksmuseum van Natuurlijke Histoire of Leiden* (Netherlands).

Figure 1. *Melanotaenia boesemani* (photo: Dr. Laurent Pouyaud)

This species is native from two distinct lakes in West Papua (i.e. Ayamaru and Uter), distant by around 30 km and separated by limestone mountains (Kadarusman, 2012). Because of its popularity, the species has been greatly exploited in its natural habitat and is currently in the verge of extinction. Since 2004, this species is included in the CITES list as an endangered species (IUCN, 2013), and theoretically only fishery products are now allowed to be exported. However, Allen reported in 2007 that around 60,000 males of *M. boesemani* were caught monthly and exported for trading (Allen, 2007). Although *Melanotaenia boesemani* has been domesticated by Indonesian farmers since 1983, the number of farms that exploit this species in Indonesia is still very low. Besides, farmers claim that there is a decline in the production, in terms of quantity and quality, such as a higher proportion of females than males per spawning, a loss of body coloration, lower growth rate and fecundity, or frequent morphological abnormalities. They interpret this as a consequence of a loss of genetic variability and inbreeding.

In this context, my work aimed at gathering new genetic information that would be useful for the aquaculture and conservation of the *Melanotaeniidae* family. Specifically, the objectives of the research were: 1) to develop new microsatellite DNA markers from the endangered *M. boesemani*, 2) to evaluate the genetic diversity of wild populations of *Melanotaenia* and refine their taxonomy, 3) to describe the geographic origins of *M. boesemani* reared by ornamental fish farmers in Indonesia, and to evaluate the genetic diversity and inbreeding pressure resulting from this domestication. My PhD work was a continuation of the PhD realized by Dr. Kadarusman in the frame of the Lengguru program. Among other activities, this

scientific program consists in describing and characterizing the genetic structure and diversity of Rainbowfishes in West Papua, through scientific expeditions jointly conducted by the Institut de Recherche pour le Développement (France), the Academy of Fisheries of Sorong (West Papua, Indonesia), the Aquaculture Research and Development Center for Ornamental Fish (Java, Indonesia), and the Indonesian Institute of Sciences (LIPI).

The present manuscript is divided into five sections: the first one consists in a bibliographic review; the next three ones present the results relating to 1) the development and succesfull validation of 12 polymorphic microsatellite DNA markers from *Melanotaenia boesemani*; 2) the use of these markers for analyzing the genetic diversity of wild populations of *Melanotaenia* from West Papua; 3) their use to investigate the genetic diversity and origin of reared strains of *M. boesemani* in Indonesia. Finally, a general discussion followed by perspectives will constitute the last section of this manuscript.

II. LITERATURE REVIEW

2.1. RAINBOWFISH DISTRIBUTION AND DIVERSITY

Rainbowfishes are very famous for ornamental trade since the 1930s (Tappin, 2010), because of their vivid coloration that is reminiscent of a rainbow. They are endemic from New Guinea and Australia. The first rainbowfish (*Melanotaenia nigrans*) was scientifically described in 1843 from the collection of freshwater fish obtained in the northern region of Australia from the King River, near Victoria Settlement - Northern Territory-Australia. In 1964, Ian Munro determined that Rainbowfishes belonged to their own family. Gerald Allen, an ichthyologist from Western Australian Museum, gave a very important contribution to the systematics of Rainbowfishes inhabiting Australia and New Guinea with the description of dozen of new species and and several new genera. There are many endemic species of rainbowfish in various systems of river and lake waters with close phylogenetic and biogeography relationships both in Australia and New Guinea (Zhu *et al*., 1994; McGuigan *et al*., 2000; Unmack, 2001). Taxonomy of *Melanotaeniidae* has therefore a long story. There were as many as 30 species that had been discovered between 1843 and 1964 but several expeditions in New Guinea over the past four decades led to the description of more than 40 new additional species (Kadarusman *et al*., 2010). *Melanotaeniidae* is believed to be part of the *Atheriniforme* order, which has evolved from the ancestors of *atherinoids* at sea (Allen, 1980).

There are currently 2 families of rainbowfish that have been described until now, and which are distributed on the islands of Australia and New Guinea (the region of Indonesia and Papua New Guinea) (Table 1): the family *Melanotaeniidae*, which includes 7 genera and 102 species, and the family *Pseudomugilidae*, composed of 18 species spread into 3 genera (Table 1). *Melanotaenia* is the largest genus of the *Melanotaeniidae* family (Ivantsoff *et al*., 1991). There are thirty-two species that are endemic from the Vogelkop Province (Nugraha *et al*., 2015). The Vogelkop province belongs to the territory of West Papua, Indonesia and biogeographically should include for Melanotaeniids the western side of Lengguru Range (i.e. all watersheds flowing to Arguni Bay), the Bomberai and the Birds Head

Peninsulas, the Birds Head Isthmus joining both peninsulas and the four major Raja Ampat islands (i.e. Misool, Batanta, Salawati, Waigeo) (Kadarusman *et al*., 2012a).

Table 1. Rainbowfish classification according to ANGFA http://rainbowfish.angfaqld.org.au/Melano.htm (download 4 September 2015)

A. ***Melanotaeniidae***

a) ***Cairnsichthys***
1. *rhombosomoides* (Nichols & Raven, 1928)

b) ***Chilatherina***
1. *alleni* Price, 1997
2. *axelrodi* Allen, 1980
3. *bleheri* Allen, 1985
4. *bulolo* (Whitley, 1938)
5. *campsi* (Whitley, 1956)
6. *crassispinosa* (Weber, 1913)
7. *fasciata* (Weber, 1913)
8. *lorentzi* (Weber, 1908)
9. *pagwiensis* Allen & Unmack, 2012
10. *pricei* Allen & Renyaan, 1996
11. *sentaniensis* (Weber, 1908)

c) ***Glossolepis***
1. *dorityi* Allen, 2001
2. *incisus* Weber, 1908
3. *kabia* (Herre, 1935)
4. *leggetti* Allen & Renyaan, 1998
5. *maculosus* Allen, 1981
6. *multisquamata* (Weber & de Beaufort, 1922)
7. *pseudoincisus* Allen & Cross, 1980
8. *ramuensis* Allen, 1985
9. *wanamensis* Allen & Kailola, 1979

d) ***Iriatherina***
1. *werneri* Meinken, 1974

e) ***Melanotaenia***
1. *affinis* (Weber, 1908)
2. *ajamaruensis* Allen & Cross, 1980
3. *albimarginata* Allen, Hadiaty, Unmack & Erdmann, 2015
4. *ammeri* Allen, Unmack & Hadiaty, 2008
5. *angfa* Allen, 1990
6. *arfakensis* Allen, 1990
7. *arguni* Kadarusman, Hadiaty & Pouyaud in Kadarusman, Hadiaty, Segura, Setiawibawa, Caruso & Pouyaud, 2012
8. *aruensis* Allen, Hadiaty, Unmack & Erdmann, 2015
9. *australis* (Castelnau, 1875)
10. *batanta* Allen & Renyaan, 1998
11. *boesemani* Allen & Cross, 1980
12. *catherinae* (de Beaufort, 1910)
13. *caerulea* Allen, 1996

14. *corona* Allen, 1982
15. *duboulayi* (Castelnau, 1878) *In* Allen G.R. (1980)
16. *eachamensis* Allen & Cross, 1982
17. *ericrobertsi* Allen, Unmack & Hadiaty, 2014
18. *exquisita* Allen, 1978
19. *fasinensis* Kadarusman, Sudarto, Paradis & Pouyaud, 2010
20. *flavipinnis* Allen, Hadiaty & Unmack, 2014
21. *fluviatilis* (Castelnau, 1878)
22. *fredericki* (Fowler, 1939)
23. *goldiei* (Macleay, 1883)
24. *gracilis* Allen, 1978
25. *herbertaxelrodi* Allen, 1980
26. *irianjaya* Allen, 1985
27. *iris* Allen, 1987
28. *japenensis* Allen & Cross, 1980
29. *kamaka* Allen & Renyaan, 1996
30. *klasioensis* Kadarusman, Hadiaty & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
31. *kokasensis* Allen, Unmack & Hadiaty, 2008
32. *kolaensis* Allen, Hadiaty, Unmack & Erdmann, 2015
33. *lacustris* Munro, 1964
34. *lakamora* Allen & Renyaan, 1996
35. *laticlavia* Allen, Unmack & Hadiaty, 2014
36. *longispina* Kadarusman, Avarre & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
37. *maccullochi* Ogilby, 1915
38. *mairasi* Allen & Hadiaty, 2011
39. *manibuii* Kadarusman, Slembrouck & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
40. *maylandi* Allen, 1982
41. *misoolensis* Allen, 1982
42. *monticola* Allen, 1980
43. *mubiensis* Allen, 1996
44. *multiradiata* Allen, Unmack & Hadiaty, 2014
45. *naramasae* Kadarusman, Nugraha & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
46. *nigrans* (Richardson, 1843)
47. *ogilbyi* Weber, 1910
48. *oktediensis* Allen & Cross, 1980
49. *papuae* Allen, 1981
50. *parkinsoni* Allen, 1980
51. *parva* Allen, 1990
52. *patoti* Weber 1907
53. *picta* Allen, Hadiaty, Unmack & Erdmann, 2015

54. *pierucciae* Allen & Renyaan, 1996
55. *pimaensis* Allen, 1980
56. *praecox* (Weber & de Beaufort, 1922)
57. *pygmaea* Allen, 1978
58. *rubripinnis* Allen & Renyaan, 1998
59. *rubrivittata* Allen, Unmack & Hadiaty, 2015
60. *rubrostriata* (Ramsay & Ogilby, 1886)
61. *rumberponensis* Kadarusman, Ogistira & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
62. *salawati* Kadarusman, Sudarto, Slembrouck & Pouyaud, 2011
63. *sembrae* Kadarusman, Carman & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
64. *senckenbergianus* Weber, 1911
65. *sexlineata* (Munro, 1964)
66. *sikuensis* Kadarusman, Sudarto & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
67. *sneideri* Allen & Hadiaty, 2013
68. *splendida inornata* (Castelnau, 1875)
69. *splendida splendida* (Peters, 1866)
70. *splendida tatei* (Zietz, 1896)
71. *susii* Kadarusman, Hubert & Pouyaud *in* Nugraha, Kadarusman, Hubert, Avarre, Hadiaty, Slembrouck, Carman, Sudarto, Ogistira & Pouyaud, 2015
72. *sylvatica* Allen, 1997
73. *synergos* Allen & Unmack, 2008
74. *trifasciata* (Rendahl, 1922)
75. *urisa* Kadarusman, Setiawibawa & Pouyaud *in* Kadarusman, Hadiaty, Segura, Setiawibawa, Caruso & Pouyaud, 2012
76. *utcheensis* McGuigan, 2001
77. *vanheurni* (Weber & de Beaufort, 1922)
78. *veoliae* Kadarusman, Caruso & Pouyaud *in* Kadarusman, Hadiaty, Segura, Setiawibawa, Caruso & Pouyaud, 2012
79. *wanoma* Kadarusman, Segura & Pouyaud *in* Kadarusman, Hadiaty, Segura, Setiawibawa, Caruso & Pouyaud, 2012
80. *wokamensis* Allen, Hadiaty, Unmack & Erdmann, 2015

f) ***Pelangia***
1. *mbutaensis* Allen, 1998

g) ***Rhadinocentrus***
1. *ornatus* Regan, 1914

B. ***Pseudomugilidae***

a) ***Kiunga***
1. *ballochi* Allen, 1983
2. *bleheri* Allen, 2004

b) ***Pseudomugil***
1. *connieae* (Allen, 1981)

2. *cyanodorsalis* Allen & Sarti, 1983
3. *furcatus* Nichols, 1955
4. *gertrudae* Weber, 1911
5. *inconspicuus* Roberts, 1978
6. *ivantsoffi* Allen & Renyaan, 1999
7. *majusculus* Ivantsoff & Allen, 1984
8. *mellis* Allen & Ivantsoff, 1982
9. *novaeguineae* Weber, 1908
10. *paludicola* Allen & Moore, 1981
11. *paskai* Allen & Ivantsoff, 1986
12. *pellucidus* Allen, Ivantsoff & Renyaan, 1998
13. *reticulatus* Allen & Ivantsoff, 1986
14. *signifer* Kner, 1866
15. *tenellus* Taylor, 1964

c) ***Scaturiginichthys***

1. *vermeilipinnis* Ivantsoff, Unmack, Saeed & Crowley, 1991

Rainbowfish family is one of the most important taxas of freshwater fish in Papua.The ancestral forms of these fish taxa has been believed to inhabit Australia since the time of Gondwana which has a close kinship with the sea herring fish, Atheriniformes (Sparks & Smith, 2004). Rainbowfish phylogenetic studies in New Guinea and Australia have been done first by McGuigan *et al*., (2000) using a small part of the cytochrome b gene (351 bp bases long). Results of this study reported that there were 3 phylogenetic clades according to 3 distinct biogeographic regions: (a) Bird's Head, (b) northern part of New Guinea, and (c) southern part of New Guinea plus Australia.

Rainbowfish live in the territory of Australia and New Guinea. In the Australian region, rainbowfish habitat spreads between latitudes of 10^{o}41'S (cape york) and 43^{o}39'S (South East cape, Tasmania) and between longitudes of 113^{o}09'E (Steep point) and 153^{o}39'E (Cape Byron). The distance between the latitude of Cape York and the South East Cape (Tasmania) is 3.680 km. The distance in longitude between Steep Point and Cape Byron is about 4.000 km.. The territory of New Guinea, with an area of around 876.800 km2, is located in the south of the equator and in the south-west Pacific, at northeast Australia. The term of New Guinea is used in the concept of freshwater fish habitat that refers to the entire island, which consists of both the Province of Indonesia (West Papua and Papua) and country of Papua New Guinea (PNG). Political boundaries follow the east meridian 141° (141°E).

New Guinea has a mountainous cordillera which runs along the central mountain which maximum height is reached at the peak Jaya (5030 meters), the highest point of Papua-New Guinea.

Monophyletic groups of strictly freshwater fishes have special significance in continental biogeography because they require freshwater for dispersal, and thus their distributions are correlated with the evolution of topography and watershed (Lundberg *et al*. 2000). In general, the fish fauna of New Guinea is closely linked to that of northern Australia. The most diverse taxa are *Eleotrididae* and *Gobiidae* of about 115 species, followed by *Melanotaeniidae* of about 102 species. Around fifty species distributed in southern New Guinea are also found in northern Australia and are limited to these two regions (Lundberg *et al*. 2000).

2.2. THE *MELANOTAENIIDAE* FAMILY

Allen (1980) proposed a generic revision of *Melanotaeniidae* based on osteological characters and recognized *Melanotaenia*, *Glossolepis* and *Chilatherina* as three closely related genera, sharing a rigid fin spine at beginning of first dorsal, anal and pelvic fins (versus soft for the other members of the family). Allen (1998) described *Pelangia* and stated that this new genus was closely related to *Glossolepis,* particularly with regards to dentition, and morphology of the premaxillary, pelvic girdle, and pectoral fin.

Melanotaenia (Gill 1862) is the most diverse genus with around 80 species and sub-species (Allen *et al.,* 2008; Allen *et al*., 2015a, b; Kadarusman *et al*., 2012; Nugraha *et al*., 2015). The genus consists of 15 taxa in Australia and 89 taxa in New Guinea. The species *Melanotaenia macculochi* (Ogilby 1915) and *M. splendida* (Peters 1866) are shared in both areas of Australia and New Guinea (Allen & Renyaan 1998).

Between 2007 and 2010, there were several expeditions conducted by IRD and Academy of Fishery Sorong in the frame of Dr. Kadarusman's PhD: Rainbowfish from West Papua (*Melanotaeniidae*): Evolution and Systematics. This study contributed to the revision of the taxonomy of rainbowfishes, especially by the

identification of new morphological criteria and the use of mitochondrial (CO1, Cyt b and D-loop) and nuclear (S7) molecular markers. Six new species of *Melanotaenia* (*M. arguni, M. fasinensis, M. salawati, M. urisa, M. veoliae,* and *M. wanoma*) were consequently described (Kadarusman *et al*. 2010; 2011; 2012b).

Based on the PhD thesis of Kadarusman (2012), the biogeography of *Melanotaeniidae* distribution consisted of 4 groups (Figure 2):

1. Western New Guinea or Vogelkop Province including the four Raja Ampat islands, the Birds Head and Bomberai Peninsulas, and the western side of the Birds Head Isthmus.
2. The Central part of the Birds Head Isthmus.
3. Northern part of New Guinea (Northern New Guinea). The region covers an area of Great Northern part and the North Eastern Province (Allen 1991). This region includes the islands of Yapen, all streams in the northern part of Weyland Plateau (Wapoga, Siriwo etc) and the north New Guinea (Memberamo, Sepik, Markham etc).
4. Southern New Guinea and Australia. This section covers the Arafuru sea to Triton Lake, Cenderawasih Bay (from Munuari to Nabire) (Figure 2).

These results gave a new view that there is a great diversity of *Melanotaeniidae* in the area of New Guinea and Australia, particularly West Papua which is the center of this family.

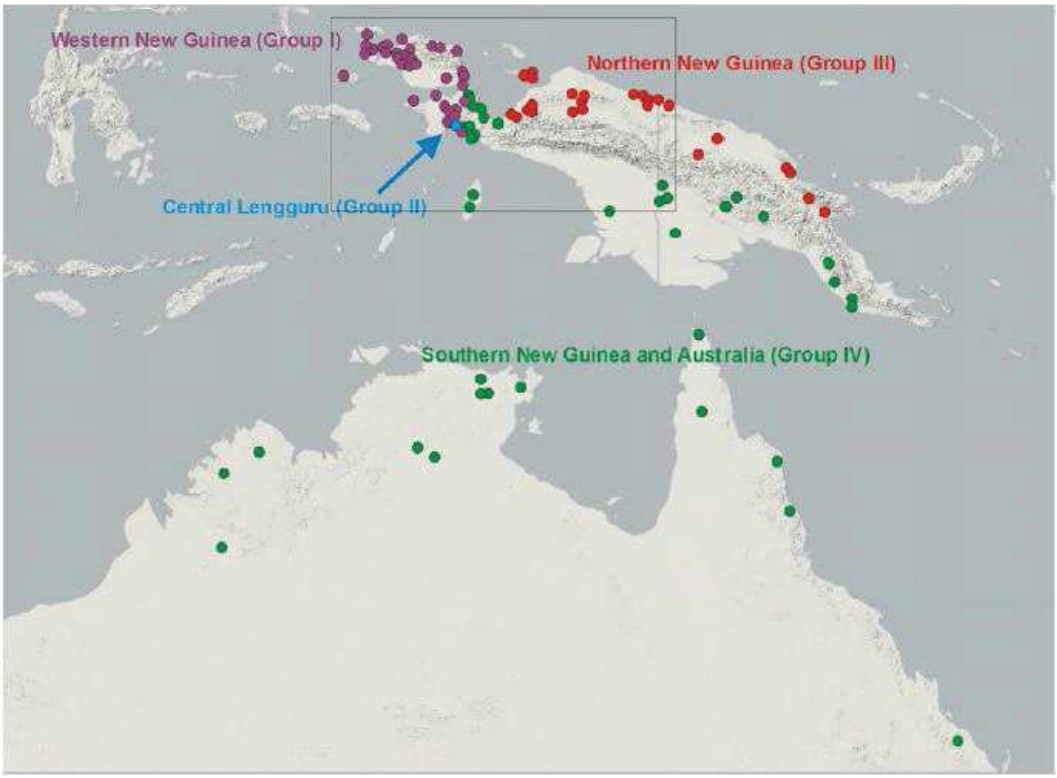


Figure 2. Map of the *Melanotaeniidae* distribution based on molecular phylogenies (Kadarusman 2012)

Specifically, in the Bird's head region of New Guinea (West Papua Province), 32 species belonging to the genus *Melanotaenia* have been described, (Nugraha *et al*. 2015). Based on species composition, Bird's head Papua is believed to be the center of rainbowfish diversity (Allen 1995).

Kadarusman *et al*. (2012a) demonstrated that species of *Melanotaenia* from the area of Bird's head are monophyletic compared to other clades. The result of the research done by Kadarusman *et al*. (2012a) showed an unpredictable diversity level, and it also showed that the diversity of *Melanotaenia* was still underestimated.

Since 2004, IUCN has identified 7 endangered species from the Bird's Head in West Papua: *Melanotaenia ajamaruensis* Allen & Cross 1980*; M. angfa* Allen 1990; *M. arfakensis* Allen 1990*; M. boesemani* Allen & Cross 1980*; M. catherinae (*de Beaufort 1910*); M. misoolensis* Allen 1982*; M. parva* Allen 1990 (IUCN 2013) (Figure 3).

Species extinction is generally caused by the introduction of alien species, excessive exploitation of species and illegal logging causing river habitat degradation and lake habitat siltation or dessication, like in the case of Kurumoi and Ayamaru lakes (Allen, 2007; Kadarusman *et al*. 2010; Tappin, 2010).

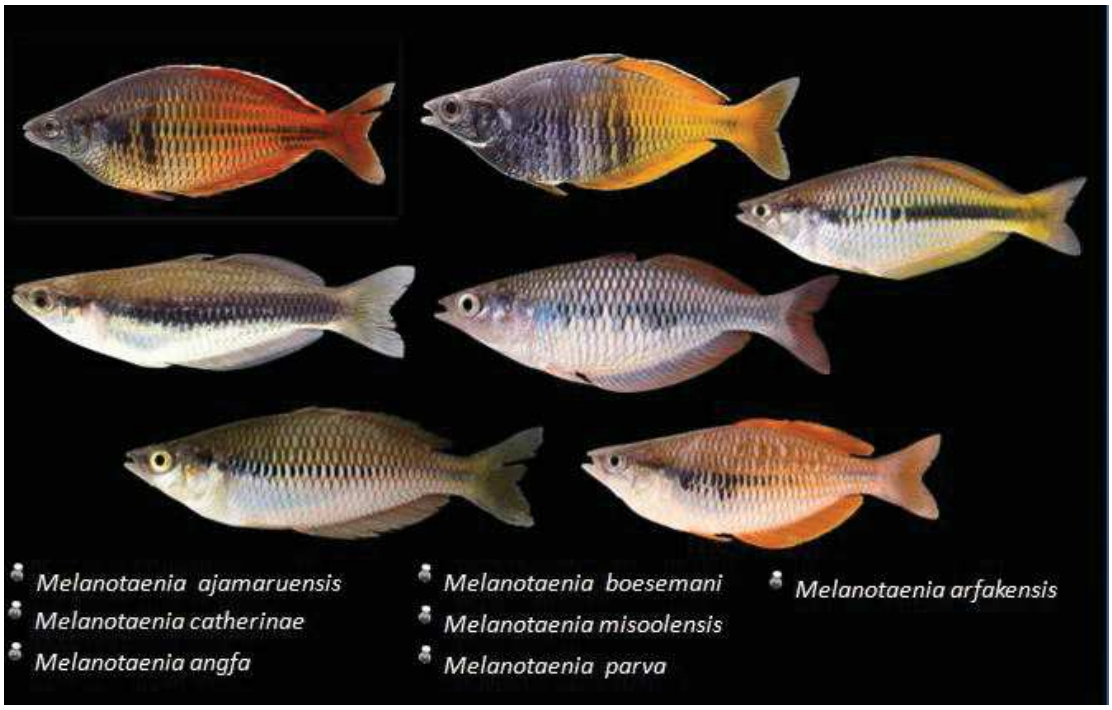


Figure 3. Seven endangered *Melanotaenia* species from Bird's head peninsula (West papua) – (Photo: Dr. Laurent Pouyaud)

Among the 102 known species of *Melanotaeniidae,* only three have been domesticated and cultivated in Indonesia, namely *Glossolepis inicus* weber 1908, *Iriatherina werneri* Meinken 1974 and *Melanotaenia boesemani* Allen & Cross 1980 (Figure 4).

Figure 4. Tree species rainbowfish, have been domesticated and cultivated in Indonesia, namely *Glossolepis inicus* weber 1908, *Iriatherina werneri* Meinken 1974 and *Melanotaenia boesemani* Allen & Cross 1980.

Melanotaenia boesemani is incontestably the most famous rainbowfish in the aquarium hobby. According to Kadarusman (2012), *M. boesemani* lives in 2 distinct locations, i.e. Ayamaru and Uter lakes. Until now, the exact origin of the strains reared in Indonesia has not been found out.

2. 3. THREATS ON THE *MELANOTAENIIDAE*

The West Papua Province (115,363 km2; 760,855 people), which encompasses the Vogelkop Peninsula and the adjacent islands of the Raja Ampat, is one of the least economically developed regions in the world. Human activities concentrate mostly in the vicinity of Sorong, Manokwari, Teminabuan and Bintuni. Papua, which includes the province of West Papua and Papua Province, is house of 50% of the Indonesian biodiversity and sinificantly contributes to Indonesia's status as one of the biologicaly richest countries in the world with Brazil (Supriatna 1999). Currently, Papua is home of 400 species of freshwater fish (Allen 1991). Until now, there have been many unspoiled areas because the access is difficult. The habitat physical topology is very steep as a result of complex geological events. This is probably the reason why new species can still be found every year (Allen 1991; Novotny *et al*., 2005).

In spite of this, the province of West Papua Indonesia is now facing new challenges. Flora and fauna in this region face a threat of extinction due to human activities such as logging and burning of forests to agricultural areas, transmigration, poaching, mining, etc. All human activities have been increasingly disturbing and destroying the habitat of flora and fauna, particularly freshwater fish. Moreover, since the 1950s, more than thirty species of freshwater fish have been introduced into the waters of New Guinea essentially for human consumption (Allen, 2007). Those species are principally the tilapias *Oreochromis mossambicus* and *O. niloticus*, the walking catfish *Clarias batrachus,* the common carp *Cyprinus carpio,* the snakehead *Channa striata*, and also other species such as *Tor putitora* and *Anabas testudineus*.

Rainbowfish species that are threatened with extinction in the region of Australia and New Guinea are: *Chilatherina axelrodi* Allen, 1980; *C. bleheri* Allen, 1985*; C. bulolo* (Whitley, 1938)*; C. sentaniensis* (Weber, 1908); *Glossolepis incisus* (Weber, 1908); *G. maculosus* Allen, 1981*; G. pseudoincisus* (Allen & Cross 1980); *G. ramuensis* Allen, 1985*; G. wanamensis* Allen & Kailola 1979*; Kingua ballochi* Allen, 1983*; K. bleheri* Allen, 2004*; Melanotaenia ajamaruensis* Allen & Cross 1980*; M. angfa* Allen, 1990 *M. arfakensis* Allen, 1990*; M. boesemani* Allen & Cross 1980*; M. catherinae (*de Beaufort, 1910*); M. corona* Allen, 1982*, M. eachamensis* Allen & Cross 1982*; M. exquisita* Allen, 1978; *M gracilis* Allen, 1978; *M. herbertaxelrodi* Allen, 1980; *M. iris* Allen, 1987*; M. lacustris* Munro, 1964*; M. maylandi* Allen, 1982*; M. misoolensis* Allen, 1982*; M. monticola* Allen, 1980*; M ogilbyi* Weber, 1910*; M. oktediensis* Allen & Cross 1980*; M. papuae* Allen, 1981*; M. parva* Allen, 1990*; M. pimaensis* Allen, 1980*; M. praecox* (Weber & de Beaufort 1922)*; M. pygmaea* Allen, 1978; *M. sexlineata* (Munro, 1964)*; M. vanheurni* (Weber & de Beaufort 1922)*; Pseudomugil connieae* (Allen, 1981); *P. furcatus* Nicholas, 1955*; P. majusculus* Ivantsoff & Allen 1984*; P. mellis* Allen & Ivantsoff 1982*; P. paskai* Allen & Ivantsoff 1986 and *Scaturiginichthys vermeilipinnis* Ivantsoff, Unmack, Saeed & Crowley 1991 (Conservation International 2002; IUCN, 2009).

The information about the biology and ecology of these fish species in their natural habitat is not much documented. An in-depth study is therefore needed, such as reproductive condition in their natural habitat, water quality, frequency of spawning and habitat preferences. Ecological research on rainbowfish families is very crucial for helping efficiently their conservation.

2. 4. HISTORY OF RAINBOWFISH AS WORLD AQUARIUM PEARLS AND AQUACULTURE

Rainbowfish from New Guinea have been imported to Australia since around the mid-1950s. They have been continuously cultivated, and their farmers had no idea that these fish will be popular for the international aquarium hobby. Some specimens were introduced, including *Melanotaenia affinis, M. goldiei, M. rubrostriata, M. papuae* and *M. sexlineata*, which were developed by hobbyists from

Australia in early 1959. In 1982, the Australian and Papua New Guinea rainbowfish were promoted through a book by Gerald Allen and Norbert Cross (Allen and Cross 1982), which resulted in the increase in popularity and curiosity about the rainbowfish species found in New Guinea and Australia. During this period, there was no significant limitation, and there was an important number of different species brought from New Guinea to Australia by private collectors, who then distributed them to hobbyists (Smith, 2007; Tappin, 2010).

The excessive trade of rainbowfish from New Guinea to Australia attracted the attention of the "Advisory Committee on Live Fish (ACOLF)". At the end of 1983, ACOLF prohibited the import of fish into Australia for all freshwater fish species from New Guinea. In the mid-1980s, aquarium fish collectors began collecting species from New Guinea, cultivating and distributing them to the international aquarium hobbyists (Smith *et al*., 2007). This trend has continued over the last two decades and has grown side by side with the increasing discovery of new species. Ichtyologists and rainbowfish hobbyists have continued to look for these fish to the inland, to collect different shapes and colors, to keep them survive in the aquarium by fulfilling all the physiological needs of the fish outside their natural habitat. However, when these fish are kept in aquarium, they do not produce bright colors as those living in their natural habitat. This resulted in the widespread sales of these fish under 5 cm in size, in which the colors have not come out well (Tappin, 2010).

Rainbowfish from Australia were domesticated for the first time as decoration in the aquarium, as reported by Albert Gale in early 1915, in his book entitled "*Aquarian nature Studies and Economic Fish farming made known the hobby of keeping Australian freshwater fishes*". At the beginning of the 1920s and 1930s, ornamental fish aquarium hobbyists were set up in major cities in Europe such as Germany. The delivery used ship, and there was no change of oxygen. When they got in the country of destination, the fish were in poor condition, but there were some survivors and they could be successfully cultivated and saved by the hobbyists. Thus this became the community of hobbyists in European countries.

During this period, these fish were known as sunfish. In January 1934, the magazine "National Geographic" published an article written by Walter H Chute, and then the director of the Shed Aquarium in Chicago USA called these as "Tropical Fish Immigrants Reveal New Nature Wonders". This raised the reference of Australian rainbowfish. The earliest record was found in the Aquarium German magazine "*Wochenschrift für Aquarien und Terrarienkunde*" in September 1931 by Erich Henzelmann, who wrote an article about '*Regenbogenfisch*' Melanotaenia nigrans. The earliest reference to the name of a rainbowfish that had been found in Australia was in a report entitled "The Aquarium and Terrarium Society of Queensland". On March 6, 1932, Amandus Rudel reported the results of his expedition and mentioned all the fish with the name "rainbowfish", while the name "Sunfish" were designated only for *Rhadinocentrus ornatus* which he described as "Moreton Island Sunfish" (Tappin, 2010).

In 1927 Amandous Rudel, a founder of Aquarium and Terrarium Society of Queensland, introduced Australian rainbowfish on the international community, by sending *Melanotaenia duboulayi* (Castelnau, 1878) to Germany. These fish were then raised in the aquarium of Berlin and then the fish were promoted to America. Amandous Rudel also introduced another rainbowfish for the international aquarium hobbyists. In 1934, he sent 12 specimens of *Melanotaenia maccullochi*, which were gathered near the river Cairns, northern Australia, and then sent to Fritz Mayer in Hamburg, Germany. Four specimens arrived and lived safely, and they developed into two pairs. These fish have become one of the most popular aquarium fish originating from Australia. This news was written in a German aquarium magazine "*Wochenschrift für Aquarien und Terrarienkunde*" in May 1935. Fritz Mayer wrote about the fish breeding techniques, translated by FH Stoye in the book "The Aquarium" in December 1936 (Tappin, 2010).

Until now, commercial species of rainbwfish in Indonesia are: *Glossolepis insicus* (from lake Sentani, Papua province), *Iriatherina werneri* (from Merauke, Papua province), *Melanotaenia boesemani* (from South Sorong, West Papua province). Until now there have been 15 groups of rainbowfish farmers in Jakarta and Bekasi region.

a) b) c) d) e) f)

Figure 5. Captive breeding of *M. boesemani* in Indonesian farms

Rainbowfish aquaculture in Indonesia began in 1983 in Mr Gusi farm. He got founder population of *M. boesemani* in his farm from exporters of ornamental fish in Jakarta, Indonesia. In that decade, *M. boesemani* became very famous because of publications, especially that by Allen and Cross (1980) on the description of *M. boesemani*. Overfishing of *M. boesemani* males then started to affect Ayamaru Lake, the natural habitat of *M. boesemani* (Allen 2007). Rainbowfish cultivation in Indonesia is carried out indoor and outdoor, involves hatcheries and grow-out ponds (Fig 5 ab), with semi-natural water bodies (Fig 5 c,d,e) and indoor aquaria installations (fig 5f). According to the quarantine authorities in Jakarta, producers

export nearly 2,500 live *M. boesemani* per week, at a price of around 1 US$ per individual. Though there are no official numbers, this is unlikely to account for the totality of *M. boesemani* that are exported worldwide from Indonesia.

1.5. MAJOR MOLECULAR MARKERS USED IN FISH GENETIC POPULATION STUDIES

A genetic marker is a measurable character of Mendelian inheritance (Liu and Cordes, 2004) and is considered ideal when polymorphic (variable between individuals), discriminatory (different related individuals), multiallelic (has many alleles on the same locus), codominant (heterozygotes are visible), not epistatic (independent of the expression of other markers), neutral (whatever the allele present at the locus, the fitness of the individual is the same), reproducible from one experiment to the next, to handle large scale and economical. The distribution of markers over the whole genome is also a criterion to remember. The main sources of molecular markers are from either a sequence polymorphism (eg: substitution, insertion, deletion) or a polymorphism number of repetitions units (microsatellites and minisatellites). There are two major types of genetic markers that are widely used to characterize fish populations, namely mitochondrial markers and microsatellite markers.

2.5.1. MITOCHONDRIAL DNA MARKERS

Mitochondrial DNA is a popular marker for study of evolution like phylogenetic inference, identification of species, phylogeography, and analysis of population structure. Characteristics of mitochondrial DNA are small genome with simple structure and organization, ubiquitous presence, high copy number, thereby easy to isolate, effective haploidy in DNA sequences, maternal inheritance, lack of recombination in introns or other non coding sequence, mosaic molecule with aster and slower evolving DNA regions allowing to design conserved primers and to

address phylogenetic questions at various taxonomic levels (Zhang & Hewiit 1996; Harrison, 1989).

For identification of species and evolution studies, maternal inheritance has important consequences, because only a fraction of the population (half if the sex ratio is 1:1) pass on their mtDNA to offspring and the effective population size for mtDNA is smaller than that for nuclear genes. In stochastic processes, it will be particularly important to determine frequencies of mtDNA genotypes. Genetic affinities defined by mtDNA genotypes reflect matriarchal phylogeny. MtDNA is suited for studying population patterns including founder populations/founder events. Because females transmit many copies of the mtDNA molecule to each offspring, a new mtDNA variant arises as mutation in a single molecule within a single cell lineage (Harrison, 1989).

MtDNA analysis has been used in three ways in this context; (1) to measure genetic variation within populations, especially ones thought to have declined recently, (2) to identify evolutionarily divergent sets of populations, including the resolution of evolutionarily significant units, and (3) to assess conservation value of populations or areas from an evolutionary or phylogenetic perspective. Mitochondrial DNA variation is more sensitive in population phenomena such as bottlenecks and hybridizations. Sex-specific differences in gene flow could also be revealed by contrasting nuclear with mitochondrial DNA. And also mtDNA is intensively studied and sequences in some parts of the molecule are highly conserved across species (Okumus & Ciftei, 2003).

A prerequisite for managing biodiversity is the identification of populations with independent evolutionary histories. Such groupings are variously categorized as species. MtDNA phylogenies can provide unique insights into population history (Avise *et al.,* 1987) and can suggest hypotheses about the boundaries of genetically divergent groups (e.g. cryptic species). Several studies about cryptic species and DNA barcoding have been succesfully implemented in the identification of previously described species. DNA barcoding studies have focused on the identification of pre-defined species (Hebert *et al.* 2003). Applying mtDNA analyses on rainbowfish, Kadarusman *et al.* (2012) succesfully described the cryptic diversity in Indo-Australian Rainbowfishes. As much as 14 species of rainbowfish have been

discovered in populations exhibiting private barcode clusters diverging from their nearest neighbor by K2P distances similar to those observed among valid species characterized by diagnostic morphological characters. DNA barcoding was not only effective for the identification of species, but it proved to be effective for the discovery of provisional cryptic diversity awaiting further screening through integrative approaches as previously predicted by other species like butterfly (Hebert *et al.* 2004), diptera (Smith *et al.* 2007) and reef fishes (Hubert *et al.* 2012) .

In spite of many advantages, mtDNA marker shows several drawbacks for genetic studies, such as non-neutrality, non-constant molecular clock, length and sequence heteroplasmy and even mitochondrial bottleneck (Stewart & Larsson, 2014).

2.5.2. MICROSATELLITE MOLECULAR MARKERS (SSR / SIMPLE SEQUENCE REPEAT)

The selection of microsatellite molecular genetic markers was found in the 1980s. They represent a unique type of tandemly repeated genomic sequences, which are abundantly distributed across genomes and demonstrate high levels of allele polymorphism. They are also called simple sequence repeats (SSRs). They are codominant markers with relatively small size, around 20 repetitions of base maximum in length, and can be easily amplified by polymerase chain reaction. They are ubiquitous in prokaryotes and eukaryotes, present even in the smallest bacterial genomes. The majority of microsatellites (30–67%) found are dinucleotides. In the genomes of vertebrates, (AC)n is the most common dinucleotide motif. It is 2.3-fold more frequent than (AT)n, the second most general type of dinucleotides (Tóth *et al*., 2000). In all vertebrates, (G+C)-rich motives (e.g., CCG, CAG) are the most common among trinucleotides. Microsatellites can be found anywhere in the genome, both in protein coding and noncoding DNA (Tóth *et al*., 2000). In eukaryotic organisms, SSRs have been shown to be in excess in noncoding regions compared to a random distribution pattern (Metzgar *et al*., 2000).

Applications of microsatellites include: genetic mapping, individual DNA identification and parentage assignment, phylogeny, population and conservation genetics, molecular epidemiology and pathology, quantitative trait loci mapping, marker-assisted selection. Also, the use of microsatellite markers may benefit the genetic dissection of complex and quantitative traits in order to map, identify and eventually clone and characterize the candidate genes controlling economically important traits. In aquaculture, SSRs represent the markers of choice for genetic monitoring of farmed stocks in view of breeding programs through the analysis of genetic variablity and pedigree structure to design beneficial crosses, select genetically improved stocks, minimize inbreeding and increase selection response (Davis & DeNise, 1998; Knibb, 2000). Microsatellite-based techniques are applied in genome scans and quantitative trait loci (QTL) mapping in natural populations to search for the genetic basis of adaptive selection and biodiversity in an increasing number of species (Rogers & Bernatchez, 2005).

Microsatellites are highly abundant in various eukaryotic genomes including all aquaculture species studied to date. In most of the vertebrate genomes, microsatellites make up a few percent of the genomes in terms of the involved base pairs, depending on the compactness of the genomes (Zhan *et al*., 2009). In fish, one microsatellite was found every 1.87 kb of DNA. For comparison, in the human genome, one microsatellite was found every 6 kb of DNA (Beckmann & Weber 1992). It is reasonable to predict that in most aquaculture fish species, one microsatellite should exist every 10 kb or less of the genomic sequences, on average (Wright 1993; Duran *et al*., 2009). Their high polymorphism, together with their PCR-based analysis, have made them one of the most popular genetic markers (Duran *et al*., 2009; Boris *et al*., 2011). Some microsatellite loci have very high numbers of alleles per locus (>20), making them very useful for applications such as parent-offspring identification in mixed populations, while others have lower numbers of alleles and may be more suited for population genetics and phylogeny (Al-Atiyat *et al*., 2012). Primers developed for one species will often cross-amplify microsatellite loci in closely related species (Boris *et al*., 2011). Genotyping of microsatellite markers is usually straightforward (Castoe *et al.,* 2010).

Microsatellite markers are co-dominant markers, which means they can distinguish heterozygous and homozygous alleles. They are usually distributed throughout the genome, and they are independent markers (Chambers & MacAvoy, 2000), neutral (any allele present at this locus has the same selective value on all individuals), and can be used from one experiment to another experiment, at an affordable cost (Goldstein & Schlötterer 1998). In the case of high allelic diversity with repetitions of mono, di, tri, tetra, penta and hexa nucleotides, the theory is based on the concept of neutrality, and the variability can differentiate individuals, populations and different species. This characteristic is very important for population genetics studies, and other genetic studies such as inbreeding pressure analysis of heredity or genetic mapping (Vartia *et al*., 2014; Bruford & Wayne 1993; Brockmann *et al*., 1994; Knapik *et al*., 1998; Goldstein *et al*., 1999; Primmer *et al*., 2000).

Microsatellites consist of four models: 1) Perfect microsatellites consist of a single continuously repeated motif without being distracted by other repeated motifs or patterns, like ctctctctctctctctct. 2) Imperfect microsatellites are microsatellites in which one or more repetitions carries base pairs that are not in accordance with the structure of repetition, like ctctctctctgtctct. 3) Interrupted microsatellites are microsatellites with the insertion of a small number of base pairs that do not fit the structure of repetition, like ctctctctctgggctctctct. 4) Compound microsatellites consist of two or more adjacent microsatellites with different repetition types, for example ctctctctctctgatgatgatgatgatgat (Goldstein and Schlötterer, 1998; Jarne and Lagoda, 1996).

Microsatellites are very abundant in eukaryotic genomes because of their process of mutations. Mutations in microsatellite loci usually involve a change in one repetition, but sometimes mutations invole multiple repetition units. There are two processes of mutation that affect the formation of microsatellite loci, namely: 1) Unequal crossing over (UCO), occurring during the process of meiosis, and 2) Slipped-strand mispairing (SSM), and occurring due to mistakes/errors during DNA replication (Goldstein and Schlötterer, 1998).

The microsatellite mutation models as addressed by Estoup and Cournet (1998) in Goldstein and Schlötterer (1998) are: 1) Infinite allele models (IAM), each mutation can create new alleles at random and assume that microsatellite mutations can create an infinite number of repeating units, and the sequence of the original alleles is not present in the population 2) Stepwise mutation models (SMM) is a gradual mutation when microsatellite mutates. They loss or gain of a single tandem repeat, and hence alleles may possibly mutate towards allele state already present in the population . 3) K-allele models (KAM): this is the classic model. In this model, all alleles have equivalent mutation probability because alleles in this model can mutate with equal probability to mutate to one of other alleles (K-1) (where in if there are 8 replications, then the mutation that has occurred is 7 replications, in which the 7 replications is the new mutation allele), 4) Two phase models (TPM) is an extension of the SMM mutation. Microsatellite mutations occur because of the increased or reduced X replications. Opportunities to microsatellite mutations involve more than one unit (Goldstein and Schlötterer, 1998; Anmarkrud *et al*., 2008).

The key feature of SSRs as molecular markers is their hypermutability and, hence, their hypervariability in species and populations. SSR grow very fast with a high mutation rate of 10^{-2} - 10^{-6} per locus per gamete per generation, when compared with point mutations in the gene encoding regions (Anmarkrud *et al*., 2008). This high level of mutation leads to a high polymorphism. The abundance of microsatellites in the genome occurs because replication slippage are not the same in sister chromatid exchange, nucleotide substitutions and duplication events as well as crossovers in the process of meiosis. Many factors might be important for the mutational processes in microsatellites, such as allele size, motif size, gender, and G/C content. Mutation pattern also depends on the specific location in the genome, *e.g* functional potential of the product when transcribed, as well as the effectiveness of various repair enzymes. In addition, microsatellite mutation rate is also influenced by the pattern of stabilization and potential secondary structure (Anmarkrud *et al*., 2008).

A very high degree of polymorphism is the most striking character of microsatellites, but not all of microsatellites are polymorphic at the same point and in the same time. Microsatellite mutations may occur during replication of chromosomes, either as part of mitosis or meiosis, and can also occur during DNA synthesis. Therefore, the frequency of microsatellite mutations increases rapidly when the cells are under stress and when the cells undergo active repair due to damage. Each mutation that occurs during meiosis or mitosis in early cleavage embryo will develop well, and if it is not selected during the development process, it will form a new microsatellite allele. In contrast, mutations that occur in the cell, as somatic mutations, will not affect other cells. There are many factors affecting the level of microsatellite mutation that makes the locus and microsatellite motif abundant (Jarne and Lagoda 1996). Besides being multiallelic and codominant at a single locus, SSRs offer several advantages over other molecular markers. For instance genetic variability can be easily detected by PCR using primers designed around the SSR repeats and by analyzing length fragments, and they are transferrable across closely related species.

Among the mechanism of this mutation, an error in the replication plays a major role in producing new alleles in the SSR loci; therefore, it increases the diversity of SSR. Replication error is the most important mechanism in the formation of SSR. During DNA replication, longer stretches of repeated units pose more of a problem to DNA polymerase than do shorter stretches making longer alleles more prone to slipped-strand mispairing. Interaction in replication errors and crossovers is an important factor to the diversity of repetition motifs and the number of SSR repetitions, such as (CA) n, (CT) n, and (CA) n (TA).

Replication error in chromosomes in each species is different depending on the rate of recombination of these species and SSR motif models in the patterns of evolution that occurs in these loci (Li *et al*., 2003). The imperfect SSR pattern makes the pattern more complex than that from the perfect SSR (Orti *'et al*., 1997). Consequently, a genome can evolve faster than other genomes, such as in the study of Li *et al.,* (2003) in *Triticum dicoccoides*. SSR sequences are more abundant and longer in vertebrates than in invertebrates, and among the vertebrates, the SSR motifs

are longer in cold-blooded species such as reptiles, amphibians and fish (Chambers and McAvoy 2000).

Transcribed microsatellites located in 5' untranslated regions (UTRs) could form specific and unusual DNA structures. In that case, the length of the repeat region could affect the translation level from the target mRNA. Microsatellites situated in the 3'-UTR could affect gene expression through their influence on the stability of transcribed products. O'Brien (1991) devided molecular markers into type I markers associated with genes of known functions and type II markers associated with anonymous genomic sequences. Microsatellites usually represent type II markers, since they are commonly located in noncoding intergenic regions. Type 1 markers are more difficult do develop (Liu *et al*., 1996). While non-gene sequences are free to mutate, causing higher levels of polymorphism, sequences within protein-coding regions generally show lower levels of polymorphism because of functional selection pressure. In addition, type II markers can be rapidly developed from SSRs isolated previously from closly related species (Bruford *et al*., 1996). Cross-species amplification of SSRs provides a possibility to superimpose the genetic information or a genetic linkage map from one species to another and therefore to use SSR markers for population genetics, parentage analysis and other applications without having to invest in the isolation of polymorphic microsatellites (Cairney *et al*., 2000). Previous research reported highly conserved flanking regions in fish (Rico *et al*., 1996) allowing cross amplification from species that diverged as long ago as 470 million years.

As previously mentioned, the various types and patterns of mutations in microsatellites cause the great diversity of patterns and lengths. This also generates what is called microsatellite homoplasy. Size homoplasy occurs when alleles show similar length in microsatellite sequences, but they are different in heredity. Thus, homoplasic microsatellites are divided into two categories: 1) microsatellite alleles that are identical in length, but not in sequence (distinguished by sequencing), 2) identical allele in both length and sequence with different evolutionary history (only detected through mutation and documented through a known pedigree) (Anmarkrud *et al*., 2008).

2.6. ISOLATION OF MICROSATELLITE MARKERS IN NON MODEL ORGANISMS

Microsatellite markers are one of the most informative and versatile DNA-based markers used in fish genetic research, but their development has traditionally been a difficult and costly process. This requires a lot of energy and a lot of work, as well as huge costs, and it takes a long time to find and design appropriate primer at non models organisms. (Telles *et al*., 2010; Vartia *et al*., 2014). Emergence of next generation sequencing (NGS) technologies has enabled the efficient identification of large numbers of microsatellites at a fraction of the cost and effort of traditional approaches. The major advantage of NGS methods is their ability to produce large amounts of sequence data from which to isolate and develop numerous genome-wide microsatellite loci. The two major methods for SSR isolation are direct shotgun sequencing of microsatellite-enriched libraries (Malausa *et al*., 2011; Guichoux *et al*., 2011). NGS are extremely high-throughput technologies that produce millions of sequences at once at a fraction of the cost of traditional Sanger methods (Shendure and Ji, 2008; Ekblom and Galindo, 2011). A direct sequencing approach has a high risk and may not detect SSR (Parchman *et al*., 2010). In this case, SSR enrichment must be considered. The technique of enriched libraries has proven very efficient and effective to isolate SSR in non model species (Malausa *et al*., 2011; Zalappa *et al*., 2012) (Figure 6).

Over the available NGS platforms, 454 pyrosequencing has shown a higher success in SSR isolation, probably due to the higher length of the reads it produces, which increase the probability of properly design SSRs primers under low to medium sequencing coverage (Zalappa *et al*., 2012). The 454 technology was the first next-generation sequencing technology released to the market, and circumvented the cloning requirement by taking advantage of a highly efficient *in vitro* DNA amplification method known as emulsion PCR. In emulsion PCR, individual DNA fragment-carrying streptavidin beads, obtained through shearing the DNA and attaching the fragments to the beads using adapters, are captured into separate emulsion droplets. The droplets act as individual amplification reactors, producing $\sim 10^7$ clonal copies of a unique DNA template per bead. Each template-containing bead is subsequently transferred into a well of a picotiter plate and the clonally

related templates are analyzed using a pyrosequencing reaction. The use of the picotiter plate allows hundreds of thousands of pyrosequencing reactions to be carried out in parallel, massively increasing the sequencing throughput. The pyrosequencing approach is a sequencing-by-synthesis technique that measures the release of inorganic pyrophosphate (PPi) by chemiluminescence. The template DNA is immobilized, and solutions of dNTPs are added one at a time; the release of PPi, whenever the complementary nucleotide is incorporated, is detectable by light produced by a chemiluminescent enzyme present in the reaction mix. The sequence of DNA template is determined from a "pyrogram," which corresponds to the order of correct nucleotides that had been incorporated. Since chemiluminescent signal intensity is proportional to the amount of pyrophosphate released and hence the number of bases incorporated, the pyrosequencing approach is prone to errors that result from incorrectly estimating the length of homopolymeric sequence stretches (i.e. indels).

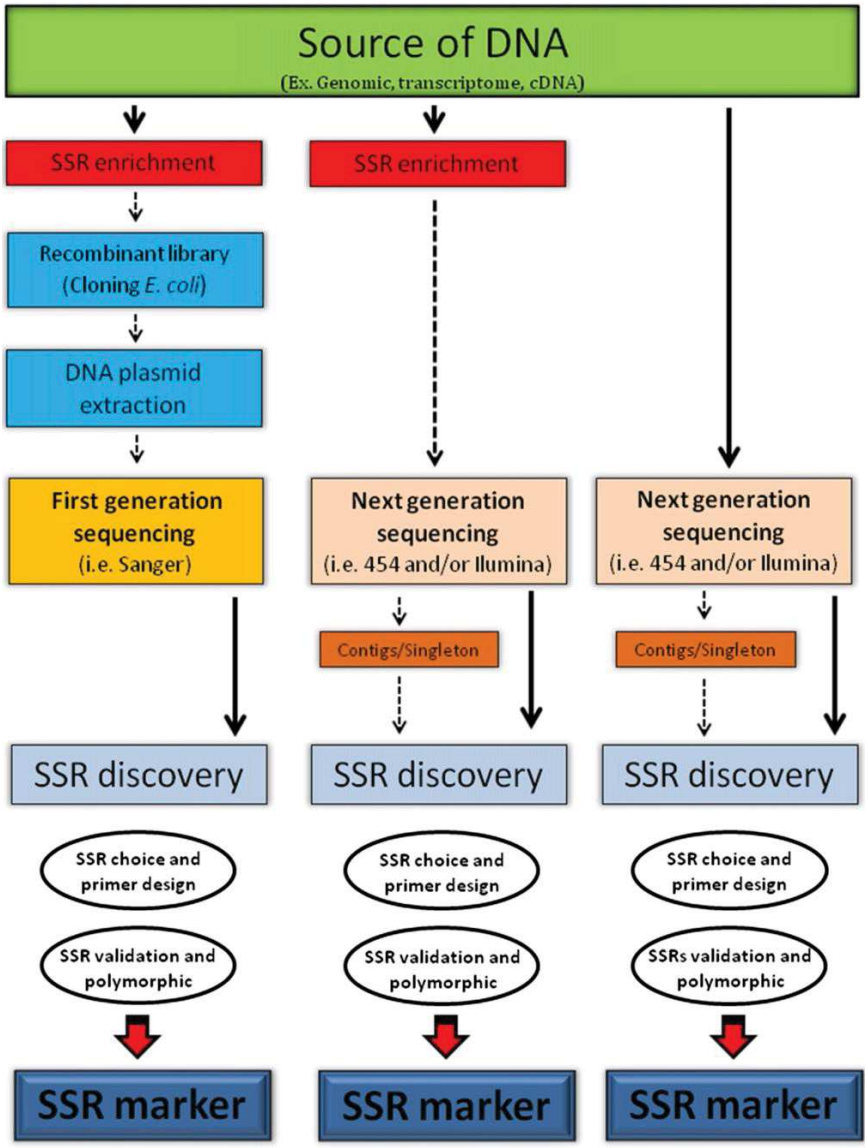


Figure 6. General flow chart for the development of simple sequence repeats (SSR) markers (reproduced from Zalappa *et al.,* 2012).

The left panel shows the previous method using Sanger sequencing. It implied an SSR enrichment followed by a cloning step to obtain a recombinant DNA library. The middle panel presents the same strategy; however, the use of NGS allows to skip the establishment of a recombinant library. Normally this method is applied for short SSR (usually dinucleotides). The right panel illustrates the direct sequencing of genomic DNA, without prior SSR enrichment. It relies on the high coverage produced by NGS platforms (millions or even billions of bases) to enable the identification of SSR motives.

Validation and microsatellite genotyping can be achieved through the use of capillary electrophoresis, in presence of fluorescent primers to detect the amplicons. These markers are then amplified in a sole PCR reaction or combined to multiplex PCRs that contain fluorescent markers with different wavelengthes (Guichoux *et al.,* 2011), like in Figure 7.

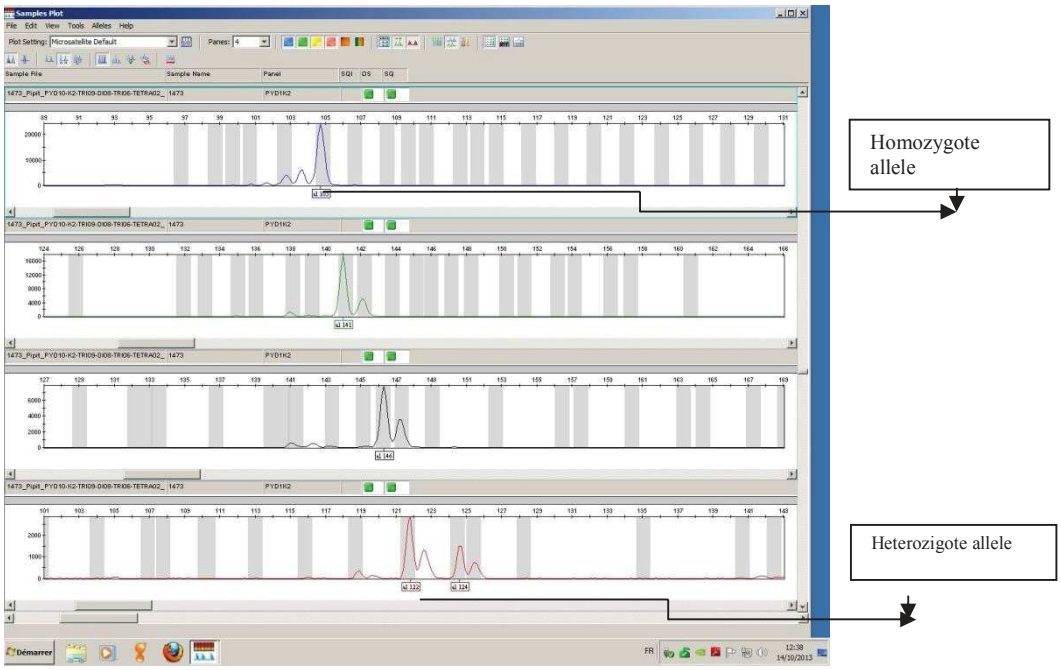


Figure 7. Allele reading of capillary electrophoresis with GeneMapper program.Illustration of a Gen Mapper view for several loci. The true allele is defined as the highest peak, and the bands before and after are considered to be stuttering bands. From top to bottom: loci Mb_tri 3, Mb_di 4, and Mb_tri4 and Mb_penta2. The 3 first ones appear homozygote in this fish specimen, while Mb_penta2 is heterozygote.

Example of microsatellite analysis of four samples that have different genotypes at the same locus. This picture shows a typical microsatellite electropherogram from the GeneMapper® Software. The number of repeats for a given locus may vary, resulting in alleles of differing lengths. The following figure shows four different dye-labeled *Melanotaenia* dinucleotide loci from four individuals. The 3 top panels illustrate 3 DNA samples that are homozygous at a specific locus (a single major peak is observed) ; the bottom panel shows a DNA sample that is heterozygous at the same locus (two major peaks are observed).

2.7. THE APPLICATION OF MICROSATELLITES IN AQUACULTURE

In the field of aquaculture, microsatellites represent workhorse markers, which are useful for the characterization of genetic stocks, broodstock selection, constructing of dense linkage maps, mapping economically important quantitative traits, identifying genes responsible for these traits and application to marker-assisted breeding programmes (Chistiakov *et al*., 2006). In fish, one microsatellite was found every 1.87 kb of DNA. For comparison, in the human genome, one microsatellite was found every 6 kb of DNA (Beckmann and Weber 1992). It is reasonable to predict that in most aquaculture fish species, one microsatellite should exist every 10 kb or less of the genomic sequences, on average (Duran *et al*., 2009).

Polymorphism can be studied from microsatellite markers which give important information to be considered in the management of fish stock (Alam and Islam 2005), population and biodiversity conservation analyses (Romana-Eguia *et al*., 2004). Boris *et al.,* (2011) have applied microsatellites to study the various genetics of hybrid red tilapia fish population, and Norris *et al.,* (1999) have applied such markers for Atlantic salmon (*Salmo salar*).

Molecular markers can be a useful tool in identifying the stock population and monitoring the potential of parent changes, as DNA fingerprint (Fjalestad *et al.,* 2003; Ahmad *et al.,* 2012). The main molecular markers that have been used widely in monitoring the variation of genetics in hatchery were derived from allozymes and microsatellite; however, the use of microsatellite markers is more efficient (Sekino *et al.,* 2002; Ramos Paredes and Grijalva- Chon 2003; Samy-Yehya *et al.,* 2012). The research on genetic variation in hatchery stock (such as in salmon) has shown the loss of genetic variation as the different results including the number of effective parental specimens, the selection of domestication or mating design (Samaradivakara *et al*., 2012). Sekino *et al*., (2002) evaluated the genetic difference among the cultivation hatchery population and wild fish population on *Paralichthys olivaceus* fish with microsatellite and mtDNA sequencing. They identified a smaller number haplotypes with mtDNA in the hatchery strains than in the wild populations.

Desvignes *et al*., (2001) studied the genetic diversity of golden fish (*Cyprinus carpio*) strains from France and Czechoslovakia using allozyme and microsatellite markers. They detected different strains of both countries with microsatellite markers. Bartfai *et al*., (2003) analyzed the whole parents from two Hungarian golden fish stockbreedings (80 and 196 fish) by using RAPD test and microsatellite analysis. The result was that the analysis of microsatellite was more informative than RAPD analysis. Microsatellite is usually the marker chosen to analyze the stock farming (Duran *et al.,* 2009; Boris *et al.,* 2011).

The monitoring of genetic changes on stock farming with microsatellite has succeeded greatly in detecting the impact of genetic changes (Zhan *et al*., 2009). The higher the detection sensitivity from the microsatellite towards the phenomenon like genetic drift and founder effect makes this marker ideal to monitor the consequence of the founder effect and propagation in farming if compared to mtDNA and allozyme markers (Duran *et al*., 2009). Microsatellite marker has been used to minimize the inbreeding on rainbow trout (Fishback *et al*., 1999). The analysis of F1 generation from *Greek gilthead sea bream broodstock* revealed the decrease of 15% in the number of alleles and the increase of 1.5% homozygosity (Magoulas, 1998). Thus, SSR marker has important application in monitoring the pressure of inbreeding. Microsatellite markers were also used to find chromosome areas that are responsible for the inbreeding pressure. Research on this is very likely to do in the future on cultivation species, in which the genitors and their offspring can be managed and tracked in close and controlled system. Moreover, this can be used to map some chromosome inheritance on a progeny and its parents (Morelli *et al.,* 2007).

Management of mixed fisheries (multiple species, different stocks) can be very tricky. To address this, genetic data have increasingly been used to investigate stock structure and stock contribution to mixed–stock fisheries. When fisheries are composed of only two different stocks, estimation of relative contribution is simple and straightforward, like for the Atlantic salmon case at West Greendland, where salmon from Europe and North America were differentiated with molecular microsatellite and minisatellite DNA markers (McConnell *et al.,* 1995). Microsatellites were found to give much higher degree of accuracy and precision than those available from allozymes (Avis, 1994).

Analysis of breeders and their progeny in programs of selective breeding uses both information about their prospective parents and relatives to increase the accuracy in parent selection and its reaction selection. In breeding, usually the number of parents used is limited and some of them fail to spawn. In this case, SSRs and other genetic markers become useful to estimate the relative success of the potential genitors. The progeny can be determined by using minisatellite or microsatellite markers after spawning (Moran *et al.,* 1996; Thompson *et al*., 1998). One of the biggest obstacles in applying the program of selective and effective fish breeding is new born offsprings that are too small to be marked physically. It is thus mandatory to keep the progeny separated from the rest of the cheptel until they become big enough to be marked. It is very expensive to do this and it is time consuming (Doyle and Herbinger, 1994). This problem can be overcome by applying DNA microsatellite markers. Of the farming result, there are a lot of tillers from fish cultivation stock that can be kept without using separated container during the initial stage. These markers have been widely used to identify relatives or progenies, and these can be used to distinguish fish in aquaculture mixed with other species (Herbinger *et al.,* 1995; Fjalestad *et al.,* 2003; Duran *et al.,* 2009; Boris *et al.,* 2011; Al-Atiyat *et al.,* 2012).

A cultivation program can start from a stockbreeding which origin is not known. The method termed “walk back” consists in tracing lineages backward (Doyle and Herbinger, 1994). The conventional way implies physical markers (tagging), and the marking of the individuals is performed when they are big enough. However, microsatellite analysis based on biopsy can be used without waiting the individual to be big enough. Some empirical studies with micosatellite loci have allowed to reconstruct the fish population genealogy and their family that are mixed when they hatch; this resulted in 99% right assignations of male and female parents and their offsprings (Norris *et al.,* 2000;. Morelli, 2007; Zhan *et al.,* 2009; Olivatti *et al.,* 2011). Doyle *et al.,* (1994) used these DNA markers to distinguish the group of cod fish family (*Gadus morhua*). One of the microsatellite marker challenges in parent management is to assess the contribution of the potential genitors in mass spawning because it often happens that many breeders fail to spawn. The use of

molecular markers will save time and cost, and the potential results can be seen clearly; moreover, this can estimate the production profit clearly and precisely.

Finally, forensics analysis in aquaculture is very important for identification of fish pedigree in selective breeding. Forensics in aquaculture aims to anticipate illegal acts like misuse of fisheries resources, misinterpretation of the content of fisheries products, illegal trading in fish or fisheries product and accidental or deliberate undesirable release or introduction into natural waters. Applications of forensics in aquaculture relate to the identification of species, populations and individuals. Microsatellite markers are very well suited to fulfill these needs (Groblera *et al*., 2005)

III. DEVELOPMENT OF NEW MICROSATELLITE MARKERS FROM *M. BOESEMANI* USING NEXT GENERATION SEQUENCING TECHNOLOGY

3.1. INTRODUCTION

Rainbowfishes of the family *Melanotaeniidae* are commonly distributed throughout New Guinea and Australia below elevations of 1500 m in most freshwater habitats including streans, lakes and swamps (Allen, 1991). Rainbowfishes are very popular ornamental fish because of their vivid colouration, placid nature and ability to thrive in captivity (Kadarusman *et al*. 2010), but many of rainbowfish have been endangered and placed on the red list of CITES since 2004 (IUCN 2013). Those are: *Melanotaenia ajamaruensis* Allen & Cross, 1980; *M. angfa* Allen, 1990; *M. arfakensis* Allen, 1990; *M. boesemani* Allen & Cross, 1980; *M. catherinae* (de Beaufort 1910); *M. misoolensis* Allen, 1982; *M. parva* Allen 1990.

The first phylogenetic relationships of the *Melanotaeniidae* was inferred by sequencing a fragment of the mitochondrial cytochrome b gene (McGuigan *et al*. 2000) and showed that *Glossolepis* genus was polyphyletic, which challenged the validity of its taxonomic status. More recently, the use of a new morphological approach and of mitochondrial and nuclear molecular markers resulted in the description of many new species (Kadarusman *et al*., 2012a; Kadarusman *et al*., 2010; Kadarusman *et al*., 2011) and showed that the diversity of rainbowfishes in West Papua was largely underestimated (Kadarusman *et al*., 2012b). Specifically, it revealed that the distribution of *Melanotaeniidae* consists of 4 monophyletic groups endemic to discrete geographic regions: Western New Guinea, Central Lengguru, Northern New Guinea and Southern New Guinea plus Australia (Kadarusman *et al*., 2012b). To date, the family consists of 105 species spread in seven genera, which are broadly distributed throughout the region. West Papua is the center of rainbowfish diversity with *Melanotaenia* genus (Allen, 1995 Allen *et al.,* 2008, Kadarusman *et al.,* 2010. McGuigan *et al.*, 2000). At the moment, 24 spesies have been described from this area : *Melanotaenia ajamaruensis* Allen & Cross, 1980; *M. ammeri* Allen *et al.*,

2008; *M. angfa* Allen, 1990; *M. arfakensis* Allen, 1990; *M. arguni* Kadaruman *et al*., 2012; *M. batanta* Allen & Renyaan, 1996; *M. boesemani* Allen & Cross, 1980; *M. catherinae* (de Beaufort, 1919); *M. ericrobertsi* Allen, Unmack & Hadiaty, 2014; *M. fasinensis* Kadarusman *et al*., 2010; *M. fredericki* (Fowler, 1939); *M. flavipinnis* Allen *et al.*, 2014; *M.irianjaya* Allen, 1985; *M. kokasensis* Allen *et al*., 2008; *M. laticlavia* Allen *et al.*, 2014; *M. misoolensis* Allen, 1982; *M. multiradiata* Allen *et al*., 2014; *M. parva* Allen, 1990; *M. salawati* Kadarusman *et al*., 2011; *M. sneideri* Allen & Hadiaty, 2013; *M. synergos* Allen & Unmack, 2008; *M. urisa* Kadarusman *et al*., 2012; *M. veoliae* Kadarusman *et al*., 2012; *M. wanoma* Kadarusman *et al.,* 2012.

Considering the high economic value of rainbowfish and the continous pressure from human towards their habitat, research on their populations in their natural habitat is very important and may lead to the discovery of new candidates for ornamental fish aquaculture. Furthermore, this can be used for conservation and endangered species rescue, as well as for cultivation comodity. In order to address these questions, new DNA microsatellite markers were developed and validated on several species of *Melanotaenia* that live in West Papua region, Indonesia.

We made the choice to use microsatellite markers to study the genetic diversity of the *Melanotaeniidae* family because the emergence of NGS has enabled to quickly and cost-effectively identify simple sequence repeats (SSR) in unstudied genomes and because they can usually be applied to closely related species (Rico *et al*., 2013). Microsatellites are markers consisting of mono-, di-, tri- or tetranucleotides tandemly repeated. Besides being generally highly polymorphic, microsatellites are generally neutral, codominant and usually abundant in the genomes of eukaryotic organisms. These characteristics are very important for population genetics studies whose theories are usually based on concepts of neutrality, independence and variability to differentiate populations and individuals.

3.2. MATERIAL AND METHODS

This research was carried out from September 2012 to October 2013. The place of the research was in the laboratory of the *'Institut des Sciences de l'Evolution de Montpellier, Université de Montpellier, France.*

3.2.1. Origin of the fish

Wild fish were captured during several expeditions in Western Papua that took place between 2007 and 2010. Microsatellite isolation was achieved from seven individuals of *Melanotaenia boesemani*. Five animals belonging to 11 other *Melanotaenia* species distributed in Western New Guinea and Central Lengguru phylogenetic groups (Kadarusman *et al*., 2012b) were also used for cross-species validation: *M. catherinae*, *M. synergos*, *M. parva*, *M. fredericki*, *M. misoolensis*, *M. salawati*, *M. batanta*, *M. veoliae*, *M. ammeri*, *M. mairasi* and *M. irianjaya*. For microsatellite experimental validation, commercial *Melanotaenia boesemani* were purchased from the French retailer Botanic (Montpellier, France) and reared in the aquatic experimental facility of ISEM (Platax, Montpellier).

3.2.2. Extraction of genomic DNA

In all cases, DNA was extracted from a small fragment (10 mg) of anal fin clip with the NucleoSpin® 96 Tissue kit (Macherey-Nagel). Tissue samples, which consisted of 1-cm^2 caudal fin preserved in absolute ethanol and stored at -20°C, were first incubated overnight at 37°C in the lysis buffer. DNA was extracted the following day according to the manufacturer's instructions, and eluted in 70 µl of the provided elution buffer. When the number of samples exceeded 24, extractions were performed with the same kit, but in 96-well plates, with a Janus automated Workstation (Perkin Elmer), in the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier).

3.2.3. Isolation of microsatellite markers

Seven individuals of *M boesemani* were used for the isolation of microsatellites markers. DNA was extracted as described above. The concentration of DNA was checked with a Nanodrop ND8000 (Thermo Scientific), and an equimolar mix of the 7 DNAs was prepared. Isolation of microsatellites was performed at Genoscreeen (Lille, France), according to the method previously described by Malausa *et al*., (2011). The mix of genomic DNA was subjected to sonication, ligation to standard adapters and purification on a Nucleofast PCR plate (Macherey-Nagel). DNA was denatured and then hybridized for 20 minute at 56°C to eight biotin-labeled oligonucleotides, the sequences of which are microsatellite motifs [(TG)10, (TC)10, (AAC)8, (AGG)8, (ACG)8, (AAG)8, (ACAT)6 and (ATCT)6]. the microsatellite-enriched DNA was then amplified using the primers corresponding to the adapters (5'-GTTTAAGGCCTAGCTAGCAGAATC-3' and 5'-GATTCTGCTAGCTAGGCCTT-3') enriched libraries were subsequently prepared and sequenced using the 454 GS FLX Titanium, following the manufacturer's protocol. Sequences were trimmed and analyzed at Genoscreen, using the QDD pipeline (Meglecz *et al*., 2010), which enables to search for simple sequence repeats (SSR) motifs and design primers in the corresponding flanking regions.

3.2.4. Amplification and size analysis of microsatellite markers

From all the microsatellite loci provided by Genoscreen, sixty one were primarily tested. The corresponding primer pairs were synthesized by Eurofins (Germany) and resuspended in water at a concentration of 100 µM. Table 2, presents the main characteristics of these primers.

Table 2. Sequence of the 61 selected microsatellite primer pairs.

No	Sequence code	Forward Primer sequence (5'-3')	Reverse primer sequence (5'-3')	Repetition Motif
1	HSOVYIT04H6V3A_A_0	CCTCAGCAGAGAACGACTGA	AACCCAAAATATTGTCTCGCA	(ac) 19
2	HSOVYIT04I00V6_A_0	TTGGTAAAACCCAGTGAAGCA	AAAGCAATTTCCTTCGGGAT	(agac) 15
3	HSOVYIT04I1G7Q_A_0*	TTTACTACACGTGGTCTTCACTTT	CGTTTTAGCCACAAACAGCC	(aatg)6
4	HSOVYIT04I1U2C_A_0	TCCTCCACATGTAACGTAGCAA	CCACGCTCAGCTTCTGTAAA	(tg) 16
5	HSOVYIT04I2DAU_A_0	GACCCAACTCTGGATAAGCG	TGGAAAGGTCCAAGGCTGTA	(tgga)9
6	HSOVYIT04I3NVN_A_0	ACCTGGGTCTTTCACCCTGT	CAGCCACATCTTTGTCAGGA	(ca)16
7	HSOVYIT04I6SQZ_A_0	TGTAATGTTCACTGACGGCTG	CAATAACATTTCGGTAGGGTCA	(ggat)9
8	HSOVYIT04I76LQ_A_0	TTCCTTCTGTGTCCTTGTTAGC	CTGGATGCTTGAATTCCTCG	(taga)8
9	HSOVYIT04I990W_A_0	AGGGCTCTCCGAAGGGATTA	GTGGGTTTCTGCTTGTGTTG	(ttcta)7
10	HSOVYIT04I9AT3_A_0	GGTGTATTCAGCCTCTTGGC	CGCCACATTTACCCAGATG	(gatg)18
11	HSOVYIT04I9MJJ_A_0	ATGCATGAGTTTCTTCTGTGGA	ATCGGACCCGTTAACACTGA	(atct)15
12	HSOVYIT04IAD7N_A_0	CCTGGCTGTGGAGGAGTGT	CAGCTATTTACTGCAGAGGATCT	(ca)19
13	HSOVYIT04IAEI4_A_0	CTTCACTATACGGCTGTTCATGT	CAGCAGGACCTGTGACTTAGA	(ac)17
14	HSOVYIT04IDFWF_A_0	GAAACATGGCAGCATAAGCA	TCATGAATGTTACTTTGTACTGAGC	(ca)15
15	HSOVYIT04IE9NT_A_0	TCTCCATGTTTTCTGTGGTGA	CACTGATCAGCCGGTCAAC	(tg) 16
16	HSOVYIT04IH6B3_A_0	ACCATGCCACGTTTAAGTCA	GGTCACCTTGTTCACCTGCT	(tgt)10
17	HSOVYIT04IHH5E_A_0	GGATCCCTTTGTAACCCAAC	GATTTCACGTACCTATCTTCAGCA	(ttatg)10
18	HSOVYIT04IHKMX_A_0	TGTCATGAAAACCTCACAGAATG	CGTTTCAATCATGGCAGAGA	(gga)13
19	HSOVYIT04IHULC_A_0	ACACTACACTGTAAAGTCACCAAGC	TGTGACTTGGACTTGCACAAA	(tatc)17
20	HSOVYIT04IIQME_A_0	TAGGGCGAGCAGAGGTTAAA	ACATTTGACTGATCCCTGGC	(ggaga)8
21	HSOVYIT04IK01K_A_0	TGCGATAGAAATTAGCGAACC	GGAGGGGTTGCGACCTAAG	(ccat)10
22	HSOVYIT04ILEWK_A_0	CGGGGAGAAAATGAGAAACA	GACAAGCACTGCACCTTCAA	(atct)16
23	HSOVYIT04ILLR5_A_0*	CAAGGCAGCAAAAGAACAAA	CAGGCCATTCCTGAGTCAAT	(ac)20
24	HSOVYIT04INQTO_A_0	CAGACCTCCTGATGTCACCAT	GAAGAAGGGACCAGGGAAAG	(atcc)12
25	HSOVYIT04INQX5_A_0	ATCGCAAACATGTGCCATAA	CAAGGGTTGCAGCCAAATAC	(tg)17
26	HSOVYIT04IOCJB_A_0*	GAAGAGTATGAAGAAGCCTTAGTCTGA	CGTCCTGCAATCTACTGTGA	(catc)18
27	HSOVYIT04IS8JB_A_0	TGAGTCAAGGGATGTCCAAA	GATGTCCCTCCATAGCCTGT	(ca)23
28	HSOVYIT04ITBI1_A_0	CAAACCTCTGCTTCGTTACTCA	TCATTTGACGTCATGGCAAC	(taga)19
29	HSOVYIT04IY6LI_A_0	TCCTACCAGATATGGGCTGC	CCACGGGACAATATTAACTGC	(gt)18
30	HSOVYIT04IZ9GS_A_0	CACCAAGCCAGGATGTGAC	GCATTTTGTTTCAGCTGTGG	(catac)8
31	HSOVYIT04IZTAQ_A_0	CTTCCCTGCTTAGGCAGCTT	TGAAACTTTAGAATCATGTTATTCAGC	(tgga)16
32	HSOVYIT04J3U3K_A_0	AACAGAAGCAGTTGCAAGTTGA	CCCAAACATTTTCTTGCCTT	(gttag)7
33	HSOVYIT04JDEPM_A_0	TCCTGCCAATGTGGTGAGTA	TAGCCTGGGTGAAGCAGAGT	(ac)18
34	HSOVYIT04JJLCQ_A_0	TCAGTGATCGTGAAGAGCAG	AGCAGGTCAGGCAGTAAACG	(caa)10
35	HSOVYIT04JNI1V_A_0	TTGACTGACGCTCCAGTTTG	CAGATGAAAATGCGGAAGGT	(atcc)19
36	HSOVYIT04JNSDY_A_0	AGGAAGAAGAGCAGGTTGGA	ACCATGGCTACCACTGCTTC	(gag)10
37	HSOVYIT04JP9IA_A_0*	TCCCACCATGCATTATTAACAC	GAACTGCAGGCTCAACACAT	(gt)18
38	HSOVYIT04JPWFS_A_0	TGACAGTAACAGGGACGATGA	AAGGCAACCTAATGTGCTGT	(atg)12
39	HSOVYIT04JQ6XV_A_0*	AAAGTGGTAAAATTTCAAAACCAA	CGATATCCACGTGTGAGGTG	(ca)23
40	HSOVYIT04JRTLS_A_0*	TGTAACGAGTGAATTTCTCCACTG	TCCACAATATTTACTGGAACTGC	(tctaa)12
41	HSOVYIT04JS706_A_0	TCTTTCCATTCAGTACCTGCG	TGACAAATTTATTTCTGTTCCACA	(gata)16
42	HSOVYIT04JT3SY_A_0	TCACCAAGGATAGTTTGTTTTGG	CTTGTTCGTGAGCTTTGTTCA	(ac)16
43	HSOVYIT04JVSN3_A_0	TCATCCACAGATCATGCACA	CACATTCACACTTCGGTGCT	(aggag)6
44	HSOVYIT04JYXHB_A_0	TGCTCTAGCCCTTAGGGAGG	CGGCCACTCGTTCATGTAT	(ggaga)13
45	HSOVYIT04JZHKE_A_0*	CTGTCAGAGTGCAGGACTGG	TCTTTTCCAGGCCAAGTGAC	(gga)8
46	concat88_A_0*	TCCTTGAATACATTCCTGCCA	CAATAATTTCAAGGAAGAGCCTTT	(ac)16
47	cons1004_2_A_0	CTGGTTGTATTGACCATGCG	CAGGCTTTGGATCAGAATCA	(ctgaac)5
48	cons1260_2_A_0*	TGGAGGATTGTAGGTCTGGG	CATCAACATAGCAATCAGTGCC	(gt)16
49	cons1347_2_A_0	TCCCCTTAAATACACCAGCG	CTGGTCTTCCTGCTCACCTT	(gag)9
50	cons1501_4_A_0	TACAAGGAGAGGAGAGGCGA	CCATGGAATGAAGATGCTGA	(ggaga)10
51	cons1666_3_A_0	CTAAAGCTAGCACACTAGCGTTCCTT	GAATAAACCTTATCTGAAAGAATGTGA	(ctt)18
52	cons1716_2_A_0*	CGTGTCTATTCCTATCGTGCC	TGGATTCACATCCTCTTGAGTG	(gag)11
53	cons220_2_A_0	TCATTTCTCACATGTCCCCA	CCTGCAGGTAGAGACCCTCA	(atc)8
54	cons2338_2_A_0	AATGATGCAGGTGAGGCG	CACCCTCGAATCATCACAGA	(tg)16
55	cons2619_3_A_0	GTGCATCCATGTGTTTCAGC	GCCTCTCCAGACTGACTTGC	(tg)17
56	cons2669_2_A_0	TTTTCATACAGGGTGCCAAA	CGCTTTGGATAAAAGCGTCT	(ctatt)5
57	cons452_2_A_0	CACTGCAGACAGAAGGCAGA	TGTACCATTCAGATGGTGGG	(gata)15
58	cons469_2_A_0	TTCAGGAGATCCTGTGTCCC	TGAAATCTGAGCCAATCGGT	(atcc)13
59	cons731_3_A_0	TGCTCCCGACCTACAGCTAC	ATGGATGGTTCTCACGGTGT	(ac)17
60	cons881_2_A_0	ATATCTCAGGAAGCCATCGG	GAATGTGGGGCTATCTCTGG	(atg)12
61	cons951_2_A_0	AACAACAGGCAGGGAGTCAC	CCAAGAGGTTGTGGTGTTCC	(atg)10

*Correspond to the 12 selected microsatellite loci

They were first tested for their ability to amplify these loci in different *Melanotaenia* species. Therefore, in order to reduce the costs, they were not fluorescently labeled. Amplifications were carried out in a GeneAmp 2400 PCR cycler (Applied Biosystems), with the Fast-Start PCR kit (Roche). Each reaction contained 5 µl of 2x Master mix (Roche), 0.4 µM of forward and reverse primers and 0.5 µl of template DNA. Cycling conditions were as follow: initial denaturation at 95°C for 4 min followed by 30 cycles of 95°C for 20 s, 56°C for 20 s and 72 °C for 30 s, and a final elongation step of 7 min at 72°C. Amplification products were analyzed by gel electrophoresis with 2% (w/v) high-melt agarose (Dutscher Scientific) in 0,5% tris-acetate EDTA and visualized with SYBR Safe DNA gel stain (Invitrogen) under a blue light transilluminator in the presence of molecular weight markers (Trackit 1 kb plus DNA ladder, Invitrogen).

Fragment size analysis was also examined by capillary electrophoresis. In this case, amplifications were performed with primers labeled with 4 different fluorescent dyes (Eurofins), in the same conditions as described above with two modifications: each reaction contained 0.1 µM of fluorescent forward primer and 0.4 µM of cold reverse primer, and the number of PCR cycles was lowered to 30. PCR products were diluted 1:50 in H2O, or sometimes 1:100 in the case of fluorescence saturation. One µl of diluted PCR from 4 distinct fluorescent couples of primers were mixed together with 13.8 µl of formamide and 0.2 µl of GeneScan™ 600LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis was run in an Applied ABI Prism® 3500 XL 24-capillary sequencer belonging to the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier).

3.2.5. Test of the Mendelian inheritance pattern of the selected microsatellite markers

Five pairs of *M. boesemani* purchased from a retailer (Botanic, Montpellier) were constituted. They were reared in distinct 60L aquariums and allowed to mate. During the two following months, aquaria were frequently checked for the presence of offspring. When available, progeny of each parental pair was collected. It consisted of 23 to 43 hatchlings, depending on the spawn size (Table 3). Juveniles

were individually transferred into 1.5 ml tubes containing absolute ethanol for further DNA extractions. Parents were anaesthetized with 0.1 mL/L Eugenol and a ~1-cm^2 piece of anal fin was collected and stored in absolute ethanol. Fish were then allowed to recover from anesthetic and were released back into their tank.

Table 3. Crossing of *M. boesemani* for microsatellite marker validation.

No	Crossing	Σ Male	Σ Female	Σ Progenies
1	Crossing 1	1	1	24
2	Crossing 2	1	1	40
3	Crossing 3	1	1	23
4	Crossing 4	1	1	32
5	Crossing 5	1	1	43

3.2.6. Microsatellite data analysis

Allele sizing and genotyping were achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems). Allelic diversity (Na) and average observed (Ho) heterozygoties were calculated using the GENETIX 4.05 software (Belkhir *et al.,* 1996). The GENETIX software is a complete program on the indices in population genetics to assess the diversity and genetic divergence. It was used to study the genetic variability in terms of heterozygosity and allelic richness rates. The markers under selection were detected from estimates of differentiation between and within samples (Fst, Weir and Cockerham, 1984) and by simulating the expected Fst under Model islands and under a mutation model (IAM) with 1,000 simulations / permutation. A significance test was applied using an exact test based on the default settings. The estimator Fis (Weir & Cockerham, 1984) was also calculated for each locus using this software. Microsatellite data were checked from Hardy-Weinberg equilibrium by estimating the significance of the multilocus inbreeding coefficient (Fis) from sets of 1000 random allelic permutations of the original dataset as implemented by GENETIX 4.05 software. The *P* value of Fis was defined as the probability to obtaining absolute values higher than or equal to the observed one under the null hypothesis (*i.e.* the individuals can be considered as the sample from a panmictic population at Hardy-Weinberg equilibrium). In order to

assess the possible presence of genotyping errors, the presence of null alleles, stuttering or large allele dropout was tested with the Micro-Checker v. 2.2.3 software (CI 95%, bootstrap simulation x1000) (Van Oosterhout *et al*., 2004). A factorial component analysis (FCA) was also conducted to assess the level of differentiation between crossing families. This analysis involves projecting the genetic distances between individuals on a set of axes that maximize the differences between populations. It was carried out using the GENETIX 4.05 software (Belkhir *et al*. 1996).

3. 3. RESULTS

3.3.1. Selection and test of candidate microsatellite markers on several *Melanotaenia* species.

Sequencing of the SSR-enriched libraries with the 454 GS-FLX Titanium sequencer resulted in a total of 88,432,250 bases and 14,922 microsatellite locus sequences from the genome of *M. boesemani*. Moreover, there were as much as 1,012 loci for which primers could be designed. From this list, we selected 61 loci according to the following criteria: the repetition sequence motif should contain 1, 2, 3, 4, 5 or 6 nucleotides ; the microsatellite motif should be pure (i.e. contain perfect reptitions); there should be at least 8 repetitions; G-C content should be comprised between 30 and 70%; product size of amplification should be comprised between 60 and 300 bp; primers should be separated from the recurring motif by at least 20 nucleotides; annealing temperatures should range between 50 and 64^{o}C (Table 1).

These 61 initially selected primer pairs were first tested for their ability to amplify *M. boesemani* DNA. On 5 tested individuals, 51 primer pairs revealed reproducible amplifications with comparable yields.

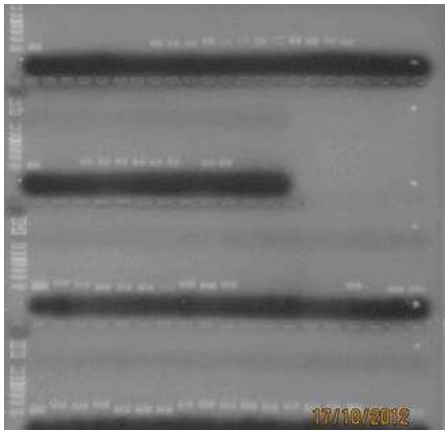
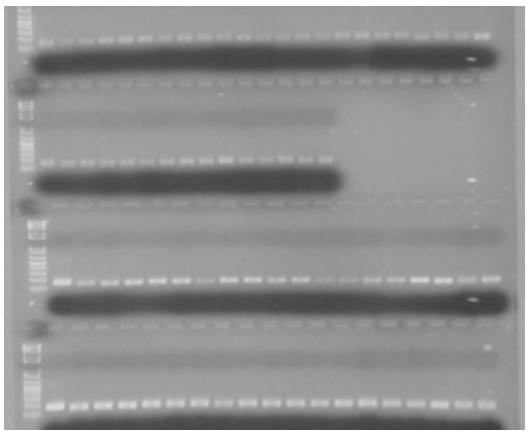
a  b 

Figure 8. Example of agarose gel results. (a) Locus that was not amplified in all tested fish; (b) locus that could be amplified in all tested fish. Polymorphism can be visualized in panel (a) as the bands do not display the same molecular weight.

From the agarose gel observations, these loci also showed polymorphism. These 51 primer pairs were subsequently tested on 11 rainbowfish species (5 specimens / species) distributed in Western New Guinea and Central Lengguru phylogenetic groups. Eventually, twelve of them successfully amplified all the tested specimens under the above-mentioned PCR conditions. The corresponding loci were renamed (Table 4) and their sequence submitted to GenBank (http://www.ncbi.nlm.nih.gov/nuccore/?term=Microsatellite+Melanotaenia+boesemani).

The 12 loci were subsequently analyzed by capillary electrophoresis. In order to reduce the costs of the genotyping experiments, they were tested in a 4-plex format as displayed in Table 3. Four PCR products were mixed together as described in the M&M section prior to capillary electrophoresis. Results obtained on a large panel of samples revealed that mixing 4 PCR products did not alter the electrophoresis quality nor the allele sizing with Peak Scanner and Gene Mapper (Figure 8).

Table 4. Name and main characteristics of the 12 microsatellite markers validated on 12 *Melanotaenia* species.

Locus name	Gene bank accession	Primer Sequence 5'-3'	Repetition Motif*	Fluorescent label	PCR Mix
Mb_di1	KF856886	F: TGAGTCAAGGGATGTCCAAA	(CA)23	5'-FAM	1
		R: GATGTCCCTCCATAGCCTGT			
Mb_di2	KF856887	F: TCCCACCATGCATTATTAACAC	(GT)18	5'-HEX	1
		R: GAACTGCAGGCTCAACACAT			
Mb_di3	KF856888	F: TGGAGGATTGTAGGTCTGGG	(GT)16	5'-ATO550	1
		R: CATCAACATAGCAATCAGTGCC			
Mb_penta1	KF856889	F: TGTAACGAGTGAATTTCTCCACTG	(TCTAA)12	5'-ATO565	1
		R: TCCACAATATTTACTGGAACTGC			
Mb_di4	KF856890	F: CAAGGCAGCAAAAGAACAAA	(AC)20	5'-FAM	2
		R: CAGGCCATTCCTGAGTCAAT			
Mb_tetra1	KF856891	F: TTTACTACACGTGGTCTTCACTTT	(AATG)6	5'-ATO550	2
		R: CGTTTTAGCCACAAACAGCC			
Mb_tri1	KF856892	F: CTGTCAGAGTGCAGGACTGG	(GGA)8	5'-ATO565	2
		R: TCTTTTCCAGGCCAAGTGAC			
Mb_tri2	KF856893	F: CGTGTCTATTCCTATCGTGCC	(GAG)11	5'-HEX	2
		R: TGGATTCACATCCTCTTGAGTG			
Mb_di5	KF856894	F: TCCTTGAATACATTCCTGCCA	(AC)16	5'-ATO565	3
		R: CAATAATTTCAAGGAAGAGCCTTT			
Mb_tetra2	KF856895	F: GAAGAGTATGAAGAAGCCTTAGTCTGA	(CATC)18	5'-HEX	3
		R: CGTCCTGCAATCTACTGTGA			
Mb_tri3	KF856896	F: TGACAGTAACAGGGACGATGA	(ATG)12	5'-ATO550	3
		R: AAGGCAACCTAATGTGCTGT			
Mb_tri4	KF856897	F: AACAACAGGCAGGGAGTCAC	(ATG)10	5'-HEX	3
		R: CCAAGAGGTTGTGGTGTTCC			

*According to the 7 genomes of *M. boesemani* used for sequencing.

3.3.2. Experimantal validation of the 12 new microsatellite markers

Microsatellites represent codominant single-locus DNA markers. For each SSR, a progeny inherits one allele from the male parent and another allele from the female parent. We therefore tested this simple inheritance pattern on 5 mate-pairs of *M. boesemani* and their progeny, in order to make sure that the new microsatellite markers did not give raise to null alleles. Results indicated that all genotypes observed in the offspring complied with those expected from the parental ones. Besides, there were no heterozygous genotypes different from the predicted ones, and genotype frequencies were similar to those expected (Table 5). The expected (He) and observed (Ho) heterozygosity values were not significantly different (Table 6).

Table 5. Allele frequencies for each breeding pair (crossing) and its corresponding offspring at the 12 microsatellite loci

Allele	Crossing and corresponding offspring														
	M1	F1	O1	M2	F2	O2	M3	F3	O3	M4	F4	O4	M5	F5	O5
Mb_di1															
95	-	-	-	-	-	-	0.50	-	0.28	-	-	-	0.50	-	0.29
97	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.21
109	-	-	-	0.50	0.50	0.55	-	-	-	-	-	-	-	-	-
113	1.00	0.50	0.67	-	0.50	0.21	-	1.00	0.50	1.00	0.50	0.70	-	0.50	0.24
117	-	0.50	0.33	0.50	-	0.24	0.50	-	0.22	-	0.50	0.30	-	-	-
119	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.26
Mb_di2															
113	-	0.50	0.33	-	-	-	0.50	1.00	-	-	-	-	-	-	-
123	-	-	-	0.50	-	0.35	-	-	-	0.50	-	0.22	-	-	-
127	-	0.50	0.17	-	-	-	-	-	-	-	-	-	-	-	-
129	-	-	-	-	0.50	0.20	-	-	-	0.50	1.00	0.78	-	0.50	0.26
133	-	-	-	0.50	-	0.15	-	-	0.72	-	-	-	-	-	-
135	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.23
139	0.50	-	0.33	-	0.50	0.30	0.50	-	0.28	-	-	-	-	-	-
141	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.50	0.51
143	0.50	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-
Mb_di3															
153	-	1.00	0.50	-	-	-	0.50	0.50	0.46	0.50	0.50	0.48	-	-	-
161	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.25
163	0.50	-	0.25	1.00	0.50	0.80	0.50	0.50	0.54	-	-	-	1.00	-	0.50
165	-	-	-	-	0.50	0.20	-	-	-	-	-	-	-	-	-
171	0.50	-	0.25	-	-	-	-	-	-	0.50	0.50	0.52	-	0.50	0.25
Mb_penta1															
160	-	-	-	-	-	-	0.50	1.00	0.65	-	-	-	0.50	-	0.29

180	-	-	-	-	-	-	-	-	-	0.50	0.50	0.59	-	-	-
185	-	-	-	-	1.00	0.50	0.50	-	0.35	-	-	-	-	0.50	0.24
190	0.50	0.50	0.56	-	-	-	-	-	-	0.50	-	0.19	-	-	-
195	0.50	-	0.23	-	-	-	-	-	-	-	-	-	-	-	-
200	-	0.50	0.21	1.00	-	0.50	-	-	-	-	0.50	0.22	0.50	-	0.21
205	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.26
Mb_di4															
112	0.50	-	0.19	0.50	-	0.26	1.00	0.50	0.76	-	0.50	0.28	-	1.00	0.50
114	0.50	0.50	0.52	-	0.50	0.23	-	0.50	0.24	1.00	0.50	0.72	-	-	-
124	-	-	-	0.50	0.50	0.51	-	-	-	-	-	-	-	-	-
140	-	0.50	0.29	-	-	-	-	-	-	-	-	-	-	-	-
164	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	0.50
Mb_tetra1															
143	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.74	-	1.00	0.50	0.50	0.50	0.47
147	-	-	-	-	-	-	0.50	-	0.26	0.50	-	0.23	0.50	0.50	0.53
157	-	-	-	-	-	-	-	-	-	0.50	-	0.27	-	-	-
Mb_tri1															
113	1.00	0.50	0.71	0.50	1.00	0.75	1.00	0.50	0.76	1.00	1.00	1.00	1.00	1.00	1.00
116	-	0.50	0.29	-	-	-	-	-	-	-	-	-	-	-	-
119	-	-	-	0.50	-	0.25	-	0.50	0.24	-	-	-	-	-	-
Mb_tri2															
138	0.50	-	0.27	-	1.00	0.50	-	-	-	0.50	0.50	0.39	-	1.00	0.50
144	-	-	-	0.50	-	0.22	-	1.00	0.50	0.50	0.50	0.61	1.00	-	0.50
147	0.50	1.00	0.73	-	-	-	0.50	-	0.43	-	-	-	-	-	-
150	-	-	-	0.50	-	0.28	0.50	-	0.07	-	-	-	-	-	-
Mb_di5															
83	-	-	-	-	-	-	0.50	-	0.28	0.50	-	0.30	0.50	0.50	0.53
89	-	-	-	0.50	-	0.26	-	-	-	-	-	-	0.50	-	0.26

91	0.50	-	0.19	-	0.50	0.24	-	0.50	0.24	-	0.50	0.27	-	0.50	0.21
95	-	-	-	-	-	-	-	0.50	0.26	-	0.50	0.23	-	-	-
97	-	0.50	0.27	0.50	-	0.24	-	-	-	0.50	-	0.20	-	-	-
105	0.50	0.50	0.54	-	0.50	0.26	0.50	-	0.22	-	-	-	-	-	-
Mb_tetra2															
180	-	-	-	-	0.50	0.29	-	-	-	-	-	-	0.50	-	0.28
188	-	-	-	-	-	-	-	0.50	0.20	-	-	-	-	-	-
192	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.22
204	-	0.50	0.25	-	0.50	0.21	1.00	-	0.50	0.50	1.00	0.77	-	1.00	0.50
208	0.50	-	0.31	-	-		-	-	-	0.50	-	0.23	-	-	-
212	0.50	0.50	0.44	1.00	-	0.50	-	-	-	-	-	-	-	-	-
256	-	-	-	-	-	-	-	0.50	0.30	-	-	-	-	-	-
Mb_tri3															
133	0.50	0.50	0.56	1.00	0.50	0.76	1.00	1.00	1.00	0.50	1.00	0.72	1.00	1.00	1.00
142	0.50	0.50	0.44	-	0.50	0.24	-	-	-	0.50	-	0.28	-	-	-
Mb_tri4															
99	0.50	0.50	0.35	1.00	0.50	0.73	0.50	0.50	0.50	0.50	-	0.23	0.50	-	0.116
102	0.50	-	0.35	-	0.50	0.28	-	0.50	0.28	0.50	1.00	0.77	-	0.50	0.186
111	-	0.50	0.29	-	-	-	0.50	-	0.22	-	-	-	0.50	0.50	0.698

M = Male, F= Female, O = offspring

Table 6. Observed (Ho) and expected (He) heterozygosity calculated on all loci for each crossing population and associated Fis

	Crossing 1	Crossing 2	Crossing 3	Crossing 4	Crossing 5
Ho ± SD	0.65 ± 0.27	0.69 ± 0.32	0.67 ± 0.30	0.56 + 0.26	0.70 ± 0.38
He ± SD	0.52 ± 0.19	0.49 ± 0.21	0.47 ± 0.19	0.43 ± 0.18	0.49 ± 0.25
Fis	NS	NS	NS	NS	NS

Based on the Fst analysis, each crossing family of *M. boesemani* represented a different population, with p value <0.001 (Tabel 7). A factorial component analysis of all crossing individuals confirmed a pattern of Mendelian inheritance (Figure 9). Consequently, it could be concluded that there was no null allele and that all microsatellite loci followed a Mendelian inheritance pattern.

Tabel 7. Fst analysis each crossing family *M. boesemani*

	Crossing 2	Crossing 3	Crossing 4	Crossing 5
Crossing 1	0.24990***	0.25688***	0.27402***	0.33310***
Crossing 2		0.25844***	0.32983***	0.28843***
Crossing 3			0.30218***	0.23617***
Crossing 4				0.29448***

*** p<0.001

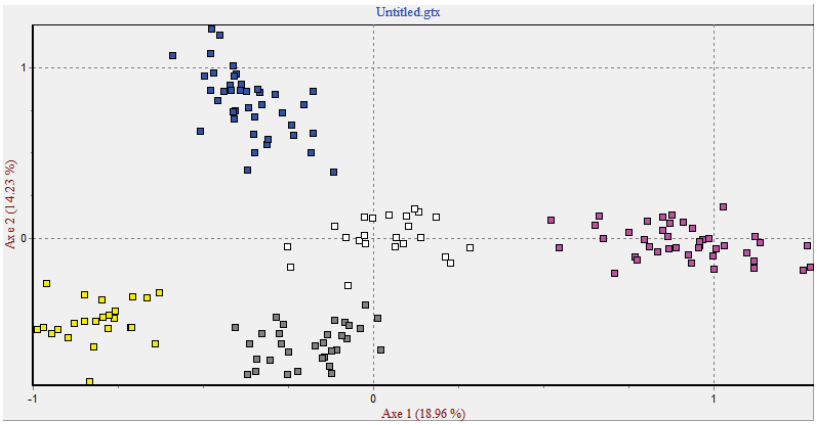


Figure 9. Factorial component analysis 5 mate-pairs of *M. Boesemani* and their progeny, ■ = Cros 1, ■ = Cross 2, □ = Cross 3, ■ = cross 4, ■ = Cross5

3.4. DISCUSSION

The use of next generation sequencing enabled very quick development of microsatellite markers in a species for which no or limited genomic resources were available. The 12 new markers described here proved efficient and polymorphic on 12 species belonging to the *Melanotaenia* genus, among which 4 species (*M. boesemani*, *M. catherinae*, *M. misoolensis* and *M. parva*) are classified in the red list of IUCN. The possible presence of null alleles in the 12 microsatellite markers was checked *in silico* and results suggested that *M. boesemani* species was not affected. However, this needed to be experimentally validated in order to definitely exclude this possibility. The cross-breeding experiments presented here showed that the 12 microsatellite markers follow a Mendelian inheritance, and ruled out the existence of null alleles due to primer misamplifications, as well as genotyping errors due to stuttering or large allele dropout. Twelve microsatellite markers had already been described for the Australian species *Melanotaenia australis* (Castelnau, 1875) (Young *et al*., 2009). Tested on 32 individuals, these loci showed a moderate polymorphism, with a number of alleles $\leqslant$ 10 for all but one of them. Two of them showed a significant deviation from Hardy–Weinberg equilibrium, suggesting the possibility of null alleles.

These results indicate that the 12 new microsatellite markers seem suitable to evaluate the genetic diversity among wild populations of rainbowfish from West Papua. Considering their effectiveness in cross-species amplification, we can reasonably expect to apply them on a larger number of species belonging to the genus *Melanotaenia*. They can also be applied to monitor the genetic changes in the aquaculture strains of M. *boesemani*, (for example genetic *M. boesemani* in Indonesian farmers is different with *M. boesemani* from Uter lake) as is already currently done for other important fish groups (Sekino *et al.,* 2002; Samy-Yehya *et al.,* 2012). Alternatively, they will also be useful in assessing the reproductive success of *M. boesemani* (Moran *et al.,* 1996; Thompson *et al.,* 1998), since Indonesian farmers claim a decline in the spawning rates and in the number of offspring per spawning (personal communication).

IV. GENETIC DIVERSITY OF WILD POPULATIONS OF *MELANOTAENIA* FROM WEST PAPUA

4.1. INTRODUCTION

Rainbowfishes of the family Melanotaeniidae are the most speciose group of pure freshwater fishes within the Australia-New Guinea region (Eschmeyer, 2014). They typically possess a compressed body covered with relatively large scales, two separate dorsal fins (the first with 3-7 spines and the second with a single spine and 6-22 segmented rays), a long-based anal fin, and no lateral line (Allen *et al*. 2008). The family is characterized by relatively small (usually less than 10 cm) and often brightly colored fish. Rainbowfishes are very popular ornamental fish with their peaceful disposition and their ease of breeding. Sexual dimorphism is often apparent and males tend to be deeper-bodied and more vividly colored than females (Allen, 1991). Generally, they are locally abundant and occupy the full spectrum of freshwater habitats including arid-zone waterholes, swamps, rainforest streams and oxbow or mountainous lakes. They are generalist omnivores and show little ecological or morphological specialization like many other freshwater species of the Australian region (Roberts, 1978; McDowall, 1981).

Melanotaeniidae occur throughout northern Australia, along the eastern costal strip until the southern Murray-Darling drainages, and also in the western part of the country. They are also found throughout the island of New Guinea below 1600 m elevation, as well as on most surrounding large islands of the western half of the mainland, including the Raja Ampat and Aru Archipelagos and Yapen Island (Allen, 1991).

According to Eschmeyer (2014) and Allen *et al.*, (2014a, b), seven genera and 105 species are currently recognised within Melanotaeniidae: *Cairnsichthys* Allen 1980 (1 species), *Chilatherina* Regan 1914 (11 species), *Glossolepis* Weber 1907 (9 species), *Iriatherina* Meinken 1974 (1 species), *Melanotaenia* Gill 1862 (81 species), *Pelangia* Allen 1998 (1 species), and *Rhadinocentrus* Regan 1914 (1 species). The greater diversity of the family occurs on the island of New Guinea

with 70 endemic species, while 13 species are restricted to Australia, and only 3 species are shared between both biogeographic entities (Allen and Hadiaty, 2013).

The tectonic history of the New Guinea region shows that the Bird's Head Peninsula is a section of the Australian craton that became separated from the main continental mass at Early Cretaceous (Pigram and Davies, 1987). At the same period, the main section of New Guinea was expanded by the collision of the northward-moving Indo-Australian plate with the westward-moving Pacific tectonic plate, and a series of smaller plates (Hamilton, 1988; Charlton, 2000; Hill & Hall, 2003). During the Late Miocene (*c.* 10-12 MYA), a northeast-southwest compression coupled with several subduction processes resulted in the uplift of the rugged Lengguru fold-and-thrust belt which unified the Bird's Head to the greater New Guinea (Bailly *et al*., 2009). This geologic episode was concomitantly marked by the uplift of the current Central Highlands that extend east-west through New Guinea (Hall, 2002). These intense tectonic and orogenic events resulted in the isolation of three major aquatic biotic provinces: northern New Guinea, the combined southern New Guinea/Australia, and the Bird's Head region including the Lengguru Range (Allen, 1991; Abell *et al*., 2008).

Kadarusman *et al.*, (2012a) assessed the DNA barcoding diversity of 53 nominal species of Melanotaeniidae throughout the Indo-Australian archipelago. This study indicated the family contains three main clades corresponding to the three biogeographic regions of New Guinea and a fourth main clade restricted to Lengguru Range.

In a recent study involving nuclear and mitochondrial markers, Unmack *et al.*, (2013) confirmed that the three main clades correlated with the three main biogeographic regions of New Guinea, but failed to evidence the fourth clade restricted to Lengguru because of a lack of DNA samples (*i.e. Melanotaenia mairasi* Allen & Hadiaty, 2011).

Kadarusman *et al*., (2012a) therefore confirmed that the *Melanotaenia* species from the Bird's Head region are monophyletic compared to the three other main clades and highlighted the presence of nearly twice as many evolutionary lineages among the 15 nominal species sampled in this area. These results revealed unexpected levels of cryptic diversity, demonstrating that the diversity of

the family has been largely underestimated. The authors concluded that the Bird's Head region should include for Melanotaeniids the western side of Lengguru Range (*i.e.* all watersheds flowing to Arguni Bay), the Bomberai and the Bird's Head Peninsulas, the Bird's Head Isthmus joining both peninsulas and the 4 major Raja Ampat Islands (*i.e.* Misool, Batanta, Salawati, Waigeo).

The Bird's Head region is a centre for rainbowfish diversity with 24 species currently described: *Melanotaenia ajamaruensis* Allen & Cross, 1980, *M. ammeri* Allen *et al*., 2008, *M. angfa* Allen, 1990, *M. arfakensis* Allen, 1990, *M. arguni* Kadarusman *et al.*, 2012, *M. batanta* Allen & Renyaan, 1996, *M. boesemani* Allen & Cross, 1980, *M. catherinae* (de Beaufort, 1910), *M. ericrobertsi* Allen *et al.,* 2014, *M. fasinensis* Kadarusman *et al*., 2010, *M. flavipinnis* Allen *et al.*, 2014, *M. fredericki* (Fowler, 1939), *M. irianjaya* Allen, 1985, *M. kokasensis* Allen *et al*., 2008, *M. laticlavia* Allen *et al.,* 2014, *M. misoolensis* Allen, 1982, *M. multiradiata* Allen *et al.,* 2014, *M. parva* Allen, 1990, *M. salawati* Kadarusman *et al.*, 2011, *M. sneideri* Allen & Hadiaty, 2013, *M. synergos* Allen & Unmack, 2008, *M. urisa* Kadarusman *et al.*, 2012, *M. veoliae* Kadarusman *et al.*, 2012, *M. wanoma* Kadarusman *et al.*, 2012.

Considering their increasing economic value and the ever-growing human pressures on their habitats, more research on the genetic variability of these fishes is needed to i) reveal cryptic diversity and occurrence of additional new *Melanotaenia* species and ii) identify candidate species for aquaculture and for conservation pur poses. To address these questions, a large number of populations from the Indo-Australian region were first genotyped with the newly developed DNA microsatellite markers. Then, a special focus was made on the Specific Bird's head region of West Papua. Whenever precise taxa could not be determined, a combination of molecular and morphologic approaches were used to assess whether morphological similar specimens from distinct locations were conspecifics or whether sufficient genetic variation and reproductive isolation existed to consider these specimens as distinct species. This led to the identification of eight new species from the Bird's Head region.

4.2. MATERIAL AND METHODS

This research was carried out from September 2012 to October 2013. The place of the research was in the laboratory of the *'Institut des Sciences de l'Evolution de montpellier, Université de Montpellier, France.*

4.2.1 Origin of the investigated fish

The samples were collected during three expeditions conducted between 2007 and 2010 in West Papua by The Akademi Perikanan Sorong, Indonesia (APSOR-KKP) The Institut de Recherche pour le Développement, Montpellier, France (IRD) and the Balai Riset Budidaya Ikan Hias in Depok, Indonesia (BRBIH-KKP). A total of 32 populations from Papua-New Guinea were analyzed. (Table 8).

Among these populations, 28 had previously been analyzed for their cytochrome oxydase I gene (COI) barcode by Kadarusman *et al* (2012a). Sampling strategy consisted to collect each population at a type locality. For most of them, between 2 and 45 individuals were captured at a given location by using a landing net (Table 8).

Because of low population density, five species (*i.e. M. ammeri*, *M. fredericki*, *M. irianjaya, M. manibuii sp. nov., M. naramasae sp. nov.*) were sampled at various localities in the same river drainage, with geographic distances varying between 1 to 5 km.

Table 8. List of all investigated populations (valid and new species in West Papua)

No	Species	Habitat	No of individuals
1	*M. ajamaruensis* Allen & Cross, 1980	Kaliwensi R, Bird's Head	30
2	*M. ammeri*Allen *et al*., 2008	Gusimawa*, Arguni Bay	20
3	*M. angfa* Allen, 1990	Yakati R*, Bird's Neck	36
4	*M. arfakensis* Allen, 1990	Prafi R*, Bird's Head	45
5	*M. arguni* Kadarusman *et al.,* 2012	Jasu Ck*, Arguni Bay	22
6	*M. batanta* Allen & Renyaan, 1996	Warmon Ck*, Batanta Is	21
7	*M. boesemani* Allen & Cross, 1980	L Ayamaru*, Bird's Head	28
8	*M. catherinae* (de Beaufort, 1919)	Waiwo Ck, Waigeo Is*	37
9	*M. ericrobertsi* Allen *et al.*, 2014	Suswa*, Bird's Head	2
10	*M. fasinensis* (Kadarusman *et al*., 2010)	Fasin Ck*, Bird's Head	40
11	*M. flavipinnis* Allen *et al., 2014*	Ifaupan Ck*. Misool Is	No samples
12	*M. fredericki* (Fowler, 1939)	Sampson R*, Bird's head	13
13	*M. irianjaya* Allen, 1985	Fruata*, Bomberai	24
14	*M. kokasensis* Allen *et al., 2008*	Kokas Ck* Bomberai	No samples
15	*M. klasioensis* Nugraha *et al.,* 2015	Klasio Ck, Bird's Head	40
16	*M. laticlavia* Allen *et al. 2014*	Aifuf Ck* Birds Head	No samples
17	*M. longispina* Nugraha *et al*., 2015	Klahfot R, Bird's Head	39
18	*M. manibuii* Nugraha *et al*., 2015	Tisbo R, Bird's Head	39
19	*M. misoolensis* Allen, 1982	Wai Tama*, Misool Is	6
20	*M. multiradiata* Allen *et al*., 2014	Moswaren*, Bird's Head	40
21	*M. naramasae* Nugraha *et al*., 2015	Naramasa R, Bird's Neck	29
22	*M. parva* Allen, 1990	L Kurumoi*, Bird's Neck	40
23	*M. rumberponensis* Nugraha *et al*., 2015	Rumberpon Is. Bird's Neck	17
24	*M. salawati* Kadarusman *et al*., 2011	Doktor Ck*, pulau Salawati	22
25	*M. sembrae* Nugraha *et al*., 2015	Sembra R, Bird's Head	15
26	*M. sikuensis* Nugraha *et al*., 2015	Siku Ck, Bird's Neck	42
27	*M. sneideri* Allen & Hadiaty, 2013	Small Ck in Kumawa*, Bomberai	No samples
28	*M. susii* Nugraha *et al*., 2015	Susi Ck, Bird's Head	31
29	*M. synergos* Allen & Unmack, 2008	Wai Bin Ck, pulau Batanta*	39
30	*M. urisa* Kadarusman *et al*., 2012	L Sewiki*, Arguni Bay	20
31	*M. veoliae* Kadarusman *et al.,* 2012	Gebiasi Ck*, Arguni Bay	24
32	*M. wanoma* Kadarusman *et al.,* 2012	Wermura Ck*, Arguni Bay	14

4.2.2. Genotyping of all populations with the 12 nuclear DNA microsatellites

Tissue samples consisted of 1-cm^2 caudal fin preserved in absolute ethanol and stored at -20°C. DNA was extracted using the NucleoSpin® 96 Tissue kit (Macherey-Nagel), according to the manufacturer's instructions, using a Janus

automated Workstation (Perkin Elmer). It was eluted in 70 µl of the provided elution buffer, and its concentration measured using a Nanodrop ND8000 spectrophotometer (Thermo Scientific). Microsatellite amplifications were performed with fluorescently labeled primers (Eurofins) and each reaction contained 5 µl of 2x Master mix (Roche), 0.1 µM of forward primer, 0.4 µM of reverse primer and 0.5 µl of template DNA. Cycling conditions were as follow: initial denaturation at 95°C for 4 min followed by 30 cycles of 95°C for 20 s, 56°C for 20 s and 72 °C for 30 s, and a final elongation step of 7 min at 72°C. Amplicon size was analyzed by capillary electrophoresis, in the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier). PCR products were diluted 1:50 in H_2O (or 1:100 in the case of fluorescence saturation). One µl of diluted PCR from 4 distinct fluorescent couples of primers were mixed together with 13.8 µl of formamide and 0.2 µl of GeneScan™ 600LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis was run in an Applied ABI Prism® 3500 XL 24 capillary sequencer. Allele sizing and genotyping were achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems).

Allelic diversity (Na), average expected (He) and average observed (Ho) heterozygoties were calculated using the GENETIX 4.05 software (Belkhir *et al.*, 1996). Locus-by-locus and multilocus Hardy-Weinberg equilibrium was tested for each species by estimating the significance of the inbreeding coefficient (Fis) from sets of 1000 random allelic permutations of the original dataset, as implemented by GENETIX 4.05 software. The *P* value of Fis was defined as the probability to obtain absolute values higher than or equal to the observed one under the null hypothesis. In order to assess the possible presence of genotyping errors, the presence of null alleles, stuttering or large allele dropout was also tested for all the analyzed loci across all the species with the Micro-Checker v. 2.2.3 software (CI 95%, bootstrap simulation x1000) (Van Oosterhout *et al.*, 2004). Genetic relationships between multilocus genotypes were assessed using factorial correspondence analysis (FCA) in GENETIX 4.05 software. Genetic structure among species was also investigated by calculating pairwise Fst values with 1000 random allelic permutations of the original dataset as implemented by GENETIX 4.05 software. Significance level of *P* value for Fst was defined as above for Fis.

4.2.3. Phylogeny of West Papua populations based on the mitochondrial DNA barcode

Table 9. *Melanotaenia* species used in barcode (COI) phylogenetic analysis and in the microsatellite marker genotyping

No	Species	Habitat	N_{COI}	GenBank # COI	No of individuals
1	*M. ajamaruensis* Allen & Cross, 1980	Kaliwensi R, Bird's Head	3	KF491175-77	30
2	*M. ammeri* Allen *et al*., 2008	Gusimawa*, Arguni Bay	3	KF491184-86	20
3	*M. angfa* Allen, 1990	Yakati R*, Bird's Neck	3	KF491293, 16-17	36
4	*M. arfakensis* Allen, 1990	Prafi R*, Bird's Head	3	KF491225, 27-28	45
5	*M. arguni* Kadarusman *et al.,* 2012	Jasu Ck*, Arguni Bay	3	KF491452-53,56	22
6	*M. batanta* Allen & Renyaan, 1996	Warmon Ck*, Batanta Is	3	KF491239, 42-43	21
7	*M. boesemani* Allen & Cross, 1980	L Ayamaru*, Bird's Head	3	KF491250-51,55	28
8	*M. catherinae* (de Beaufort, 1919)	Waiwo Ck, Waigeo Is*	3	KF491258-60	37
9	*M. ericrobertsi* Allen *et al*., 2014	Suswa*, Bird's Head	2	KF491410-11	2
10	*M. fasinensis* (Kadarusman *et al*., 2010)	Fasin Ck*, Bird's Head	2	KF491265,68	40
11	*M. flavipinnis Allen et al., 2014*	Ifaupan Ck*. Misool Is		No samples	
12	*M. fredericki* (Fowler, 1939)	Sampson R*, Bird's head	3	KF491284,88-89	13
13	*M. irianjaya* Allen, 1985	Fruata*, Bomberai	3	KF491340,46-47	24
14	*M. kokasensis Allen et al., 2008*	Kokas Ck* Bomberai		No samples	
15	*M. klasioensis* Nugraha *et al*., 2015	Klasio Ck, Bird's Head	3	KF491398-99,03	40
16	*M. laticlavia Allen et al., 2014*	Aifuf Ck* Birds Head		No samples	
17	*M. longispina* Nugraha *et al*., 2015	Klahfot R, Bird's Head	2	KF491415-16	39
18	*M. manibuii* Nugraha *et al*., 2015	Tisbo R, Bird's Head	3	KF491440-41,44	39
19	*M. misoolensis* Allen, 1982	Wai Tama*, Misool Is	3	KF491366-68	6
20	*M. multiradiata* Allen *et al*., 2014	Moswaren*, Bird's Head	3	KF491422,26-27	40
21	*M. naramasae* Nugraha *et al*., 2015	Naramasa R, Bird's Neck	3	KF491213-15	29
22	*M. parva* Allen, 1990	L Kurumoi*, Bird's Neck	3	KF491376-78	40
23	*M. rumberponensis* Nugraha *et al*., 2015	Rumberpon Is. Bird's Neck	3	KF491207,09-10	17
24	*M. salawati* Kadarusman *et al*., 2011	Doktor Ck*, pulau Salawati	3	KF491390,94-95	22
25	*M. sembrae* Nugraha *et al*., 2015	Sembra R, Bird's Head	3	KF491435-37	15
26	*M. sikuensis* Nugraha *et al*., 2015	Siku Ck, Bird's Neck	3	KF491203-05	42
27	*M. sneideri* Allen & Hadiaty, 2013	Small Ck in Kumawa*, Bomberai		No samples	
28	*M. susii* Nugraha *et al*., 2015	Susi Ck, Bird's Head	3	KF491417,19,21	31
29	*M. synergos* Allen & Unmack, 2008	Wai Bin Ck, pulau Batanta*	3	KF491466-68	39
30	*M. urisa* Kadarusman *et al*., 2012	L Sewiki*, Arguni Bay	3	KF491404-06	20
31	*M. veoliae* Kadarusman *et al.,* 2012	Gebiasi Ck*, Arguni Bay	3	KF491447-49	24
32	*M. wanoma* Kadarusman *et al.,* 2012	Wermura Ck*, Arguni Bay	3	KF491459-61	14

*Type locality; N_{COI} sample size for barcode; GenBank #COI, accesion number for barcode sequence; N_{SSR}, sample size for microsatellite markers.

Partial 650-bp sequences from the 5' region of the cytochrome oxydase I gene (COI) were obtained from GenBank for 20 valid species and 8 undetermined taxa (according to Kadarusman *et al.*, 2012a) for which DNA was available (Table 9). Phylogenetic analysis of the COI data set was inferred with neighbor joining (NJ) and maximum likelihood (ML) methods in MEGA5 (Tamura *et al.,* 2011). The Kimura 2-parameter (K2P) substitution model was used for the NJ analysis with 1000 bootstrap replicates to infer evolutionary distances. We also performed ML analyses with 500 bootstrap replicates using the Kimura 2-parameter model with a discrete Gamma distribution (K2P+G), which was the most appropriate nucleotide substitution model for our dataset based on the Bayesian Information Criterion (BIC) in the ML model selection feature of MEGA5.

4.2.4. Specimen deposition and new species description

Specimens of the new species were deposited at the Museum Zoologicum Bogoriense (MZB, Indonesia). The methods for counting and measuring were derived from Allen & Cross (1980) with some modifications and additions (Kadarusman *et al.*, 2010). Measurements were performed with digital dial callipers under lightening monocular lens (x2) and counts were made under lightening binocular lenses (x4). Measurements were made on the left side and were expressed to the nearest 0.1 mm. All proportions were expressed as percentage of the standard length. Counts and measurements were performed by Dr. Kadarusman, as this part of my thesis is the continuation of the work initiated by this latter in the frame of his PhD.

The comparative material included the 20 valid species described from the Bird's Head region with their COI barcode. This material consisted of type specimens and additional specimens caught at the type localities during the three expeditions (2007-2008-2010). These additional specimens were deposited at MZB, RMNH and MNHN.

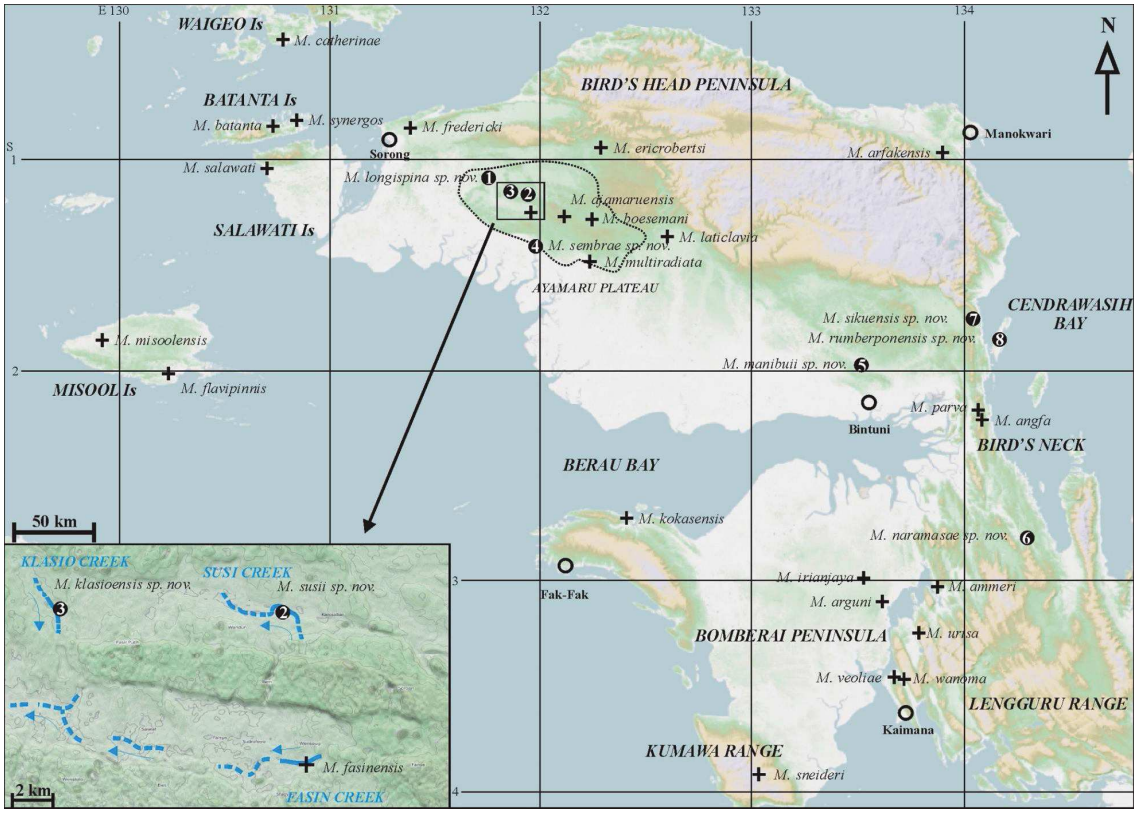


Figure 10. Type localities of the 24 valid *Melanotaenia* species and the 8 new species from the Bird's Head region

The geographic locations of the new species and comparative material are presented in Figure 10 and are as follow:

1. *Melanotaenia ajamaruensis*. – Indonesia, West Papua. RMNH 28068 (holotype), 77.9 mm SL, Ajamaru; RMNH 28069-71 (paratypes), 7 spms (57.0-63.8 mm SL), collected with holotype by Boeseman, 4-6 March 1955; MZB 17692-94, 16 spms (62.5-96.5 mm SL), MNHN 2009-1617, 6 spms (55.5-66.5 mm SL), Kaliwensi River at Soroang village, 1°15.073'S, 132°08.156'E, a karst resurgence connected to Lake Ayamaru by subterranean outlets, Kadarusman, Krenak, Paradis & Pouyaud, 22-23 May 2007.
2. *Melanotaenia ammeri*. – Indonesia, West Papua. MZB 16455 (holotype), 82 mm SL; Gusimawa village; MZB 16456 (paratypes), 4 spms (55.9-71.0 mm SL), collected with holotype by Allen & Ammer, 12 January 2008; MZB 17709, 9 spms (62.4-101.8 mm SL), collected at type locality, 3°02.438'S, 133°52.844'E, Lemauk & Pouyaud, 4 November 2010.

3. *Melanotaenia angfa*. – Indonesia, West Papua. MZB 17698, 3 spms (69.2-86.2 mm SL), MNHN 2009-1620, 5 spms (62.1-68.6 mm SL), RMNH.PISC.35675, 5 spms (59.5-62.6 mm SL), Pondok Creek, type locality, River Yakati, 2°11.067'S, 134°05.584'E, Kadarusman, Ajambua, Sumanta & Pouyaud, 11 April 2008.
4. *Melanotaenia arfakensis*. – Indonesia, West Papua. MZB 17702, 3 spms (68.7-76.9 mm SL), MNHN 2009-1622, 4 spms (60.7-70.6 mm SL), RMNH.PISC.35677, 3 spms (60.9-71.3 mm SL), Supsan Creek, 0°58.376'S, 133°54.964'E, a tributary of Prafi River (type locality), Kadarusman, Ajambua, Paradis & Pouyaud, 30 May 2007.
5. *Melanotaenia arguni*. – Indonesia, West Papua. MZB 17712 (holotype), male, 54.9 mm SL, Egerwara village, Jasu Creek, 3°05.292'S, 133°37.879'E; MZB 17713 (paratypes), 6 spms (58.7-67.6 mm SL), MNHN 2010-0029 (paratypes), 4 spms (59.1-64.3 mm SL), RMNH.PISC.35876 (paratypes), 4 spms (58.9-72.6 mm SL), collected with holotype by Kadarusman, Ruwe & Wamburie, 1st Nov. 2010.
6. *Melanotaenia batanta*. – Indonesia, West Papua. MZB 17705-06, 5 spms (88.2-99.2 mm SL), MNHN 2009-1625, 5 spms (79.5-107.9 mm SL), RMNH.PISC.35678, 5 spms (73.9-110.3 mm SL), type locality, Warmon stream, 0°50.256'S, 130°43.287'E, Bouhris, Sumanta & Pouyaud, 30 April 2008.
7. *Melanotaenia boesemani*. – Indonesia, West Papua. RMNH 28061 (holotype), 67.2 mm SL, Ajamaru Lakes, Boeseman, 4 March 1955; RMNH 28067 (paratypes), 2 spms (63.8-86.9 mm SL), same data as holotype; MZB 17691, 6 spms (54.7-60.7 mm SL) and MNHN 2009-1616, 4 spms (61.8-66.0 mm SL), Tiwit Creek, approx. 1 km N of Ayamaru L, 1°15.463'S, 132°14.939'E, Kadarusman, Slembrouck & Pouyaud, 22 May 2007.
8. *Melanotaenia catherinae*. – Indonesia, West Papua. MZB 17703, 4 spms (65.4-79.8 mm SL) and MNHN 2009-1623, 4 spms (58.1-67.1 mm SL), Waiwo Creek on Waigeo island (type locality), approx. 5.5 km N of Saonek Island, 0°25.060'S, 130°46.462'E, Kadarusman & Pouyaud, 14 May 2007.

9. *Melanotaenia fasinensis*. – Indonesia, West Papua. MZB 17700 (holotype), male, 108.5 mm SL, Ween village, Fasin Creek, 1°13.856'S, 131°58.186'E; MZB 17701 (paratypes), 4 spms (91.0-120.2 mm SL), MNHN 2009-1627 (paratypes), 4 spms (77.1-90.8 mm SL), RMNH.PISC.35680, 4 spms (69.9-78.5 mm SL), collected with holotype by Sumanta, Krenak, Kadarusman, Paradis & Pouyaud, 24 May 2007.
10. *Melanotaenia fredericki*. – Indonesia, West Papua. MZB 17695, 4 spms (65.0-71.9 mm SL), RMNH.PISC.35673, 4 spms (65.0-81.5 mm SL), MNHN 2009-1618, 4 spms (55.7-71.7 mm SL), Sampson River (or Warsamson), type locality, 0°49.361'S, 131°24.193'E, Kadarusman & Paradis, 13 June 2007.
11. *Melanotaenia irianjaya*. – Indonesia, West Papua. MZB 4952 (holotype), 58.8 mm SL, Fruata; MZB 4953 (paratypes), 3 spms (39.6-57.8 mm SL), same data as holotype; MZB 17708, 10 spms (67.7-102.6 mm SL), Wat Creek, 2°58.576'S, 133°27.971'E, about 7 km from type locality, Kadarusman, 1st November 2010.
12. *Melanotaenia kokasensis.* – Indonesia, West Papua. MZB 16453 (holotype), 57.2 mm SL, Kokas, Allen & Ammer, 16 Januari 2008: MZB 16454 (paratypes), 2 spms (49.1 – 53.7 mm SL), same data as for holotype
13. *Melanotaenia misoolensis*. – Indonesia, West Papua. MZB 17707, 3 spms (59.8-77.4 mm SL), RMNH.PISC.35679, 3 spms (46.1-48.9 mm SL), MNHN 2009-1626, 4 spms (54.0-58.4 mm SL), Wai Tama, type locality, 1°50.978'S, 129°54.654'E, Ajambua, Slembrouck, Sumanta & Pouyaud, 28 April 2008.
14. *Melanotaenia multiradiata.* – Indonesia, West Papua. MZB 20033, 14 spms (64.1-89.9 mm SL), Waren R, at Moswaren, type locality, 1°29.158'S, 132°13.596'E, Sumanta, Ajamba, Kadarusman, Slembrouck & Pouyaud, 23 April 2008.
15. *Melanotaenia parva*. – Indonesia, West Papua. MZB 17699, 2 spms (65.4-67.0 mm SL), MNHN 2009-1621, 3 spms (57.7-62.0 mm SL), RMNH.PISC.35676, 3 spms (50.7-58.6 mm SL), Lake Kurumoi, 2°09.761'S, 134°05.155'E, collected at type locality by Kadarusman, Paradis, Sumanta, Pouyaud; 7-8 June 2007.

16. *Melanotaenia salawati*. – Indonesia, West Papua. MZB 17710 (holotype), male, 88.7 mm SL, Waipule village, Doktor Creek, 1°01.128'S, 130°41.407'E; MZB 17711 (paratypes), 5 spms (71.4-91.6 mm SL), MNHN 2010-0030 (paratypes), 5 spms (68.0-83.6 mm SL), RMNH.PISC.35875, 5 spms (77.1-82.2 mm SL), collected with holotype by Sumanta, Ajambua, Slembrouck & Pouyaud, 29 April 2008.
17. *Melanotaenia synergos*. – Indonesia, West Papua. MZB 17704, 2 spms (61.8-67.1 mm SL) and MNHN 2009-1624, 3 spms (57.7-63.5 mm SL), a small creek on north-eastern Batanta Island, less than 3 km from type locality, 0°50.288'S, 130°47.227'E, Kadarusman, Sumanta & Pouyaud, 15 May 2007.
18. *Melanotaenia urisa*. – Indonesia, West Papua. MZB 17715 (holotype), male, 71.4 mm SL, Sewiki Lake, 3°15.061'S, 133°47.735'E; MZB 20025 (paratypes), 6 spms (60.6-71.0 mm SL), MNHN 2010-0031 (paratypes), 5 spms (63.7-87.8 mm SL), RMNH.PISC.35877 (paratypes), 5 spms (57.4-62.0 mm SL), collected with holotype by Ajambua, Lemauk, Legendre & Pouyaud, 1st Nov. 2010.
19. *Melanotaenia veoliae.* – Indonesia, West Papua. MZB 20026 (holotype), male, 92.3 mm SL, Gebiasi Creek, 3°27.607'S, 133°41. 071'E; MZB 20027 (paratypes), 5 spms (62.8-87.2 mm SL), MNHN 2010-0032, 5 spms (53.9-84.5 mm SL), RMNH. PISC 35878, 5 spms (64.5-65.0 mm SL), collected with holotype by Segura, Caruso,Setiawibawa, Sauri, Suruwaki, 7 Nov. 2010.
20. *Melanotaenia wanoma*. – Indonesia, West Papua. MZB 20028 (holotype), male, 71.9 mm SL, Wermura Creek, 3°28.261'S, 133°42.770'E; MZB 20029 (paratypes), 5 spms (62.8-72.8 mm SL), MNHN 2010-0033, 6 spms (59.6-65.0 mm SL), RMNH.PISC.35879, 6 spms (61.0-71.9 mm SL), collected with holotype by Segura, Caruso, Setiawibawa, Sauri, Suruwaki, 7 Nov. 2010.
21. *Melanotaenia klasioensis* (species newly described from the present work). MZB 20034, male, 83.9 mm SL, 1°09.005'S, 131°51.927'E, a bridge on Klasio Creek near Malabolo village and on the road from Sorong to Ajamaru (km110), at the frontier post between Sorong and Sorong Selatan Regencies, Papua Barat, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 24 April 2008.

22. *Melanotaenia longispina* (species newly described from the present work)**.** MZB 22152, female, 78.7 mm SL, 1°04.160'S, 131°45.595'E, a bridge on Klahfot R., near Saluk village, km92 on the road from Sorong to Teminabuan, Sorong Regency, Papua Barat, Indonesia. Sumanta, Krenak, Kadarusman, Paradis & Pouyaud, 24 April 2008.
23. *Melanotaenia susii* (species newly described from the present work)**.** MZB 22155, male, 114.1 mm SL, 1°09.114'S, 131°57.665'E, a bridge on Susi Creek, on the road from Sorong to Ajamaru, 12 km after the frontier Post between Sorong and Sorong Selatan Regencies, Papua Barat, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 24 April 2008
24. *Melanotaenia sembrae* (species newly described from the present work)**.** – MZB 22158, male, 87.3 mm SL, 1°24.667'S, 131°59.231'E, Sembra R. near Teminabuan, Sorong Selatan Regency, Papua Barat, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 23 April 2008.
25. *Melanotaenia manibuii* (species newly described from the present work)**.** – MZB 22166, male, 103.7 mm SL, 1°56.330'S, 133°31.036'E, Tisbo R. near Bintuni, Papua Barat, Indonesia. Sumanta, Ajambua, Kadarusman, 4 June 2007.
26. *Melanotaenia naramasae* (species newly described from the present work)**.** – MZB 22160, male, 99.1 mm SL, 2°48.440'S, 134°19.965'E, Naramasa R., Bird's Neck Isthmus, Papua Barat, Indonesia. Kadarusman, Ogistira, Sumanta & Pouyaud, 21 April 2009.
27. *Melanotaenia rumberponensis* (species newly described from the present work)**.** – MZB 22162, male, 65.2 mm SL, 1°51.238'S, 134°09.489'E, Rumberpon Island, Cendrawasih Bay, Bird's Neck Isthmus, Papua Barat, Indonesia. Kadarusman, Ogistira, Ajambua, Sumanta & Pouyaud, 5 April 2008.
28. *Melanotaenia sikuensis* (species newly described from the present work)**.** – MZB 22164, male, 91.0 mm SL, 1°46.784'S, 134°04.790'E, Siku Creek, Bird's Neck Isthmus, Papua Barat, Indonesia. Kadarusman, Paradis, Ajambua, Sumanta & Pouyaud, 2 June 2007.

4.3. RESULTS

4.3.1. Analysis of all the wild populations with the microsatellite markers

Twelve microsatellite loci were successfully amplified on 775 specimens (28 species) including those analyzed by their COI DNA barcode analysis (Table 9 Fig. 17). Characteristics of these loci are presented in table 10. All microsatellite loci revealed polymorphic with a number of alleles per-locus ranging between 8 (locus Mb_tri2) to 53 (Mb_di2). Total number of alleles per-species (Table 10) indicate that *M. ericrobertsi* and *M. veoliae* are the species with the lowest level of polymorphism (13 and 17 alleles) while *M. multiradiata* and the new species *M. longispina* are the most polymorphic species (124 and 133 alleles respectively). Average observed heterozygosity per species ranged between 0.042 and 0.743 (Table 11). Most the species were at panmictic equibrium according to non significant multilocus FIS values (Table12). Only five species (i.e. *M. ammeri*, *M. fredericki*, *M. irianjaya*, *M. manibuii*, *M. naramasa*) showed a significant deviation from hardy-Weinberg equilibrium. No evidence for null alleles, scoring errors due to stuttering and for large allele dropout was revealed on the entire data by using Micro-Checker software. It is important to remind here that the five species presenting significant multilocus Fis values are a mix of individuals sampled at various locations along the same river drainage. These results therefore suggest the possible occurrence of genetically differentiated subpopulations patchily distributed in the same river and must be taken in consideration in future sampling strategies or population monitoring of *Melanotaenia* species.

Table 10. Characteristics of the microsatellite markers on *Melanotaenia boesemani* and on the 28 cross-amplified species.

Locus name	Bank Gene code	Primer sequences 5'-3'	Repetition Motif [a]	S[b]	S[c]	A[d]	A[e]
Mb_di1	KF85688	F: TGAGTCAAGGGATGTCCAAA	(CA)23	089-139	085-151	14	34
		R: GATGTCCCTCCATAGCCTGT					
Mb_di2	KF856887	F: TCCCACCATGCATTATTAACAC	(GT)18	113-145	111-227	14	53
		R: GAACTGCAGGCTCAACACAT					
Mb_di3	KF856888	F: TGGAGGATTGTAGGTCTGGG	(GT)16	153-173	148-231	8	48
		R: CATCAACATAGCAATCAGTGCC					
Mb_penta1	KF856889	F: TGTAACGAGTGAATTTCTCCACTG	(TCTAA)12	160-200	150-240	8	18
		R: TCCACAATATTTACTGGAACTGC					
Mb_di4	KF856890	F: CAAGGCAGCAAAAGAACAAA	(AC)20	112-152	098-172	15	38
		R: CAGGCCATTCCTGAGTCAAT					
Mb_tetra1	KF856891	F: TTTACTACACGTGGTCTTCACTTT	(AATG)6	135-157	125-217	7	33
		R: CGTTTTAGCCACAAACAGCC					
Mb_tri1	KF856892	F: CTGTCAGAGTGCAGGACTGG	(GGA)8	107-119	101-128	4	10
		R: TCTTTTCCAGGCCAAGTGAC					
Mb_tri2	KF856893	F: CGTGTCTATTCCTATCGTGCC	(GAG)11	138-150	132-153	4	8
		R: TGGATTCACATCCTCTTGAGTG					
Mb_di5	KF856894	F: TCCTTGAATACATTCCTGCCA	(AC)16	085-099	077-129	8	23
		R: CAATAATTTCAAGGAAGAGCCTTT					
Mb_tetra2	KF856895	F: GAAGAGTATGAAGAAGCCTTAGTCTGA	(CATC)18	172-252	136-324	18	39
		R: CGTCCTGCAATCTACTGTGA					
Mb_tri3	KF856896	F: TGACAGTAACAGGGACGATGA	(ATG)12	133-154	111-163	3	16
		R: AAGGCAACCTAATGTGCTGT					
Mb_tri4	KF856897	F: AACAACAGGCAGGGAGTCAC	(ATG)10	099-114	098-132	4	13
		R: CCAAGAGGTTGTGGTGTTCC					

[a] According to the *M. boesemani* DNA used to construct the genomic DNA library
[b] Allele size range (bp) for *M. boesemani*
[c] Allele size range (bp) for all 28 species
[d] Allele number for *M. boesemani*
[e] Allele number for all 28 species

Table 11. Sample size, allele diversity and average observed (Ho) and expected (He) heterozygosity per population across loci

Population name	Locus Mb_												Na	Ho	He	N
	di 1	di2	di3	penta1	di4	tetra1	tri1	tri2	di5	tetra2	tri3	tri4				
M. ajamaruensis	9	12	8	7	10	6	3	5	5	11	3	3	82	0.6699	0.6357	30
M. ammeri	2	2	2	3	1	1	1	1	2	1	1	1	18	0.1046	0.1557	20
M. angfa	1	2	2	2	1	3	1	1	3	2	1	1	20	0.2448	0.234	35
M. arfakensis	6	3	12	9	1	5	2	1	2	11	6	1	59	0.4037	0.3975	45
M. arguni	8	7	11	7	5	10	5	4	5	10	5	3	80	0.6769	0.6939	21
M. batanta	1	8	1	1	1	2	1	1	1	9	1	1	28	0.1583	0.1402	21
M. boesemani	14	14	8	8	15	7	4	4	8	18	3	4	107	0.6815	0.678	28
M. catherinae	2	1	1	3	1	2	1	1	1	2	1	3	19	0.1464	0.1436	37
M. ericrobertsi	1	1	1	2	1	1	1	1	1	1	1	1	13	0.042	0.042	2
M. fasinensis	4	5	9	3	4	6	1	1	2	8	1	1	45	0.3646	0.3508	40
M. fredericki	7	19	17	10	3	12	3	6	12	9	4	4	106	0.6568	0.7105	13
M. irianjaya	13	14	17	9	6	14	8	5	10	12	7	5	120	0.7082	0.7546	24
M. misoolensis	4	5	3	5	4	5	2	1	4	4	2	1	40	0.5611	0.5129	5
M. multiradiata	13	10	23	9	19	13	3	3	4	18	7	2	124	0.6083	0.6127	40
M. parva	5	5	8	6	1	5	2	2	3	4	2	1	44	0.3863	0.3796	40
M. *salawati*	1	5	6	5	8	4	2	2	3	6	2	1	45	0.3	0.3	22
M. synergos	2	1	1	3	1	3	1	1	2	3	2	1	21	0.1906	0.1863	39
M. urisa	1	3	2	5	2	5	1	3	1	4	2	4	33	0.3762	0.3974	19
M. veoliae	1	1	1	2	1	1	3	1	3	1	1	1	17	0.1267	0.1184	24
M. wanoma	1	2	4	4	3	5	3	3	2	3	1	2	33	0.3869	0.3688	14
M. klasioensis	1	15	6	2	3	4	2	1	2	3	1	1	41	0.3104	0.3046	40
M. longispina	18	9	16	9	15	17	3	6	13	14	7	6	133	0.7433	0.7312	39
M. naramasa	15	15	18	10	12	8	4	6	6	9	6	1	110	0.6377	0.6952	29
M. rumberponensis	2	2	2	3	1	4	1	2	3	1	2	1	24	0.216	0.2099	17
M. sembrae	2	15	7	5	2	5	4	2	3	13	2	6	66	0.5375	0.5117	15
M. sikuensis	2	3	2	2	1	3	2	2	1	1	2	1	22	0.1491	0.1579	42
M. susii	1	13	14	5	6	11	3	1	1	5	3	1	64	0.4325	0.4358	31
M. manibuii	4	19	19	8	2	9	5	2	5	3	6	2	84	0.5652	0.6085	39
Number allele per locus	141	211	221	147	130	171	72	69	108	186	82	60				

Na= Number of alleles ; N=number of samples

Table 12. Locus by locus and multilocus Fis values for each population.

Population	Mb_di1	Mb_di2	Mb_di3	Mb_penta1	Mb_di4	Mb_tetra1	Mb_tri1	Mb_tri2	Mb_di5	Mb_tetra2	Mb_tri3	Mb_tri4	Multilocus
M. ajamaruensis	0.062	-0.046	-0.084	0.042	-0.104	-0.134	0.112	-0.128	-0.014	-0.036	-0.051	-0.072	-0.037
M. ammeri	0.088	-0.027	0.363***	0.347*	------	------	------	------	0.697***	------	------	------	0.351***
M. angfa	------	0.043	0.023	-0.087	------	-0.066	------	------	-0.028	-0.058	------	------	-0.032
M. arfakensis	0.072	-0.026	-0.036	0.019	------	0.036	0	------	-0.159	-0.018	0.055	------	-0.004
M. arguni	0.043	0.059	-0.033	0.191*	0.157*	0.013	0.077	-0.08	-0.032	0.054	0.129	-0.086	0.048
M. batanta	------	-0.087	------	------	------	-0.026	------	------	------	-0.132	------	------	-0.104
M. boesemani	0.099	0.074	-0.048	-0.137	-0.054	0.06	-0.11	0.112	0.002	0.106*	-0.105	0.017	0.013
M. catherinae	0.094	------	------	-0.078	------	0.107	------	------	------	-0.108	------	-0.115	-0.006
M. ericrobertsi	------	------	------	------	------	------	------	------	------	------	------	------	------
M. fasinensis	-0.113	0.127	-0.106	0.117	-0.247	-0.023	------	------	0.483**	-0.016	------	------	-0.027
M.fredericki	0.073	-0.023	-0.027	0.317**	0.024	0.016	0.232*	0.235*	0.309***	0.069	0.191	0.02	0.118***
M. irianjaya	0.140*	-0.013	-0.115	0.132*	0.173	0.015	-0.045	-0.032	0.159*	-0.077	0.685***	0.133	0.084**
M. misoolensis	0.172	0.059	0.733***	0.059	0.070	0.143*	-0.429	------	-0.136	-0.351	-0.667	------	0.008
M. multiradiata	0.103	0.115	-0.037	-0.034	-0.018	-0.079	-0.017	-0.039	0.186	-0.058	0.191	-0.04	0.020
M. parva	-0.045	-0.143	-0.074	0.088	------	0.028	-0.054	-0.068	0.047	0.089	0	------	-0.005
M. salawati	------	-0.012	0.005	-0.066	-0.118	-0.046	-0.05	-0.068	-0.028	-0.105	-0.024	------	-0.061
M. synergos	-0.019	------	------	-0.146	------	-0.033	------	------	-0.056	0.160	0	------	-0.010
M. urisa	------	0.030	-0.261	0.131	0.309*	0.090	------	0.034	------	-0.080	0.370*	0.186	0.080
M. veoliae	------	------	------	-0.007	------	------	-0.206	------	0.051	------	------	------	-0.048
M. wanoma	------	0.447**	0.274	-0.093	-0.056	-0.191	0.145	0.077	-0.083	-0.273	------	-0.040	-0.012
M. klasioensis	------	-0.046	-0.046	-0.026	0.06	0.047	-0.164	------	0.093	-0.041	------	------	-0.006
M. longispina	0.032	-0.06	-0.095	0.001	-0.013	-0.002	-0.118	0.118	-0.02	-0.055	0.124	-0.026	-0.003
M. naramasae	-0.024	0.265**	0.131**	-0.068	0.006	0.02	-0.017	0.023	0.361**	0.384**	0.06	------	0.100***
M. rumberponensis	-0.151	0	-0.143	-0.076	------	-0.08	------	-0.103	0.477	------	-0.231	------	0.001
M. sembrae	-0.077	0.085	-0.02	-0.096	-0.368	-0.117	-0.064	1.000	-0.05	-0.138	0.451	0.085	-0.015
M. sikuensis	0.079	0.145	-0.025	0.027	------	-0.006	-0.025	0.071	------	------	-0.053	------	0.068
M. susii	------	-0.046	0.042	0.143	0.052	-0.033	-0.039	------	------	-0.104	0.17	------	0.024
M. manibuii	-0.145	0.015	-0.009	0.119*	0	-0.038	0.128*	-0.116	0.086	0.059	0.433**	0.316	0.084**

- Indicates a monomorphic locus, *p<0.05, **p<0.01, **P<0.001

4.3.2. Confirmation by the microsatellite markers of valid species and 8 new *Melanotaenia* species from West Papua

In order to assess the genetic relationships between the predicted new species and all other closely related nominal species (according to the COI phylogeny), several factorial correspondence analyses (FCA) were computed using the genotypes obtained with the 12 microsatellite markers. The results were as follow:

(1) The three new species *M. klasioensis*, *M. longispina* and *M. susii* were genetically differentiated from each other as well as from *M. arfakensis* and all the remaining species that constitute the "Central Ayamaru Plateau" cluster (Fig. 11). All pairwise Fst values showed highly significant genetic divergence ($p < 0.001$) among these species (Table 13). Pairwise Fst values between the 3 new species (i.e. *M. klasioensis*, *M. longispina*, *M. susii*) were comprised between 0.254 and 0.418. While the new species *M. klasioensis* showed a low genetic distance with *M. fasinensis* on the COI (Fig. 17), the microsatellite data indicated that these two species constitute distinct genetic entities (Fig. 11) characterised by a significant Fst value of 0.633 (Table 13). The same results were observed between *M. susii* and *M. ericrobertsi* and between *M. longispina* and all other species belonging to the "Central Ayamaru Plateau" cluster.

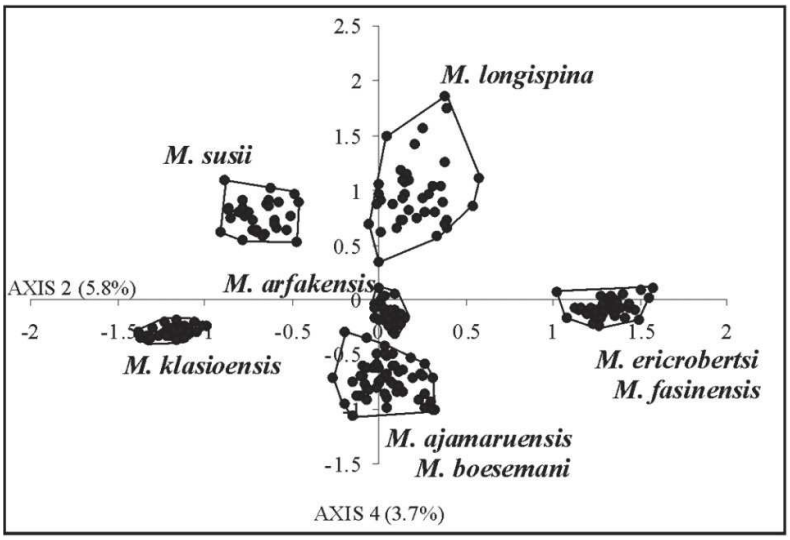


Figure 11. Factorial correspondence analysis of genotypes (255 individuals; 12 loci) from all the species belonging to the phylogenetic cluster "Central Ayamaru Plateau" and to *M. arfakensis*. Projection on axis 2 and axis 4 segregating the new species *M. klasioensis, M. longispina* and *M. susii* with other species.

(2) The FCA presented in figure 19 shows that the new species *M. sembra* is genetically differentiated from its sister species *M. multiradiata* (cluster "Southern Ayamaru Plateau"; Fig. 12). This new species was also genetically differentiated from *M. arfakensis* and from all nominal species (i.e. *M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi*, *M. fasinensis*) belonging to the cluster "Central Ayamaru Plateau". All pairwise Fst values computed between these species were highly significant ($p < 0.001$) and are given in table 14.

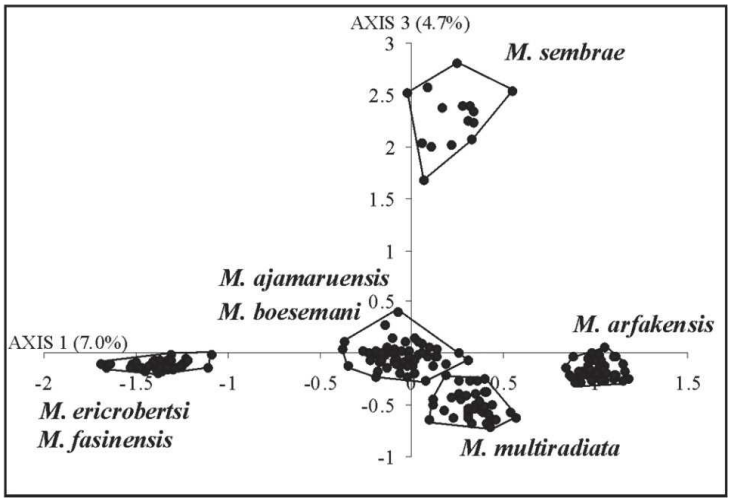


Figure 12. Factorial correspondence analysis of genotypes (200 individuals; 12 loci) from all valid species belonging to cluster "Southern Ayamaru Plateau", to cluster "Central Ayamaru Plateau" and to *M. arfakensis*. Projection on axis 1 and axis 3 segregating *M. sembra sp. nov*, with other species.

(3) The FCA in figure 13 shows that the new species *M. manibuii* is genetically separated from *M. arfakensis*, *M. multiradiata*, *M. fredericki* and from all the species distributed in the Raja Ampat Islands (i.e. *M. batanta*, *M. misoolensis*, *M. salawati*). In addition, the FCA presented in figure 14 indicates that *M. manibuii* is genetically separated from all nominal species composing the cluster "Central Ayamaru Plateau".

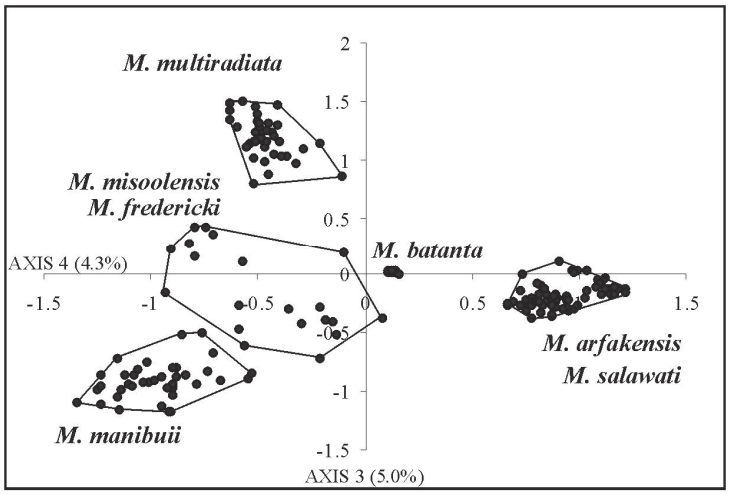


Figure 13. Factorial correspondence analysis of genotypes (186 individuals; 12 loci) from *M. manibuii, M. arfakensis, M. multiradiata, M. fredericki, M. batanta, M. misoolensis, M. salawati.* Projection on axis 3 and axis 4 segregating *M. manibui* sp. nov with other species

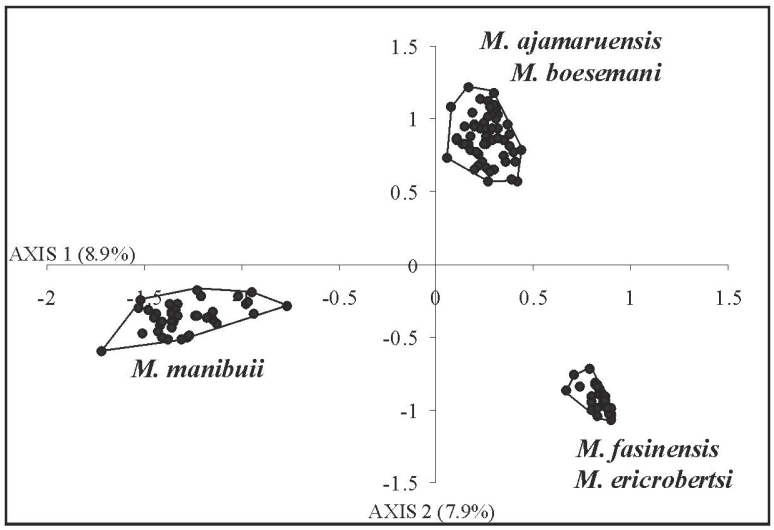


Figure 14. Factorial correspondence analysis of genotypes (139 individuals; 12 loci) from *M. manibuii*, and all valid species belonging to the cluster 'Central Ayamaru Plateau". Projection on axis 1 and axis 2 segregating *M. manibuii* sp.nov with other species

(4) The FCA obtained from the genotypes of the five new species (Figure 15) confirmed that *M. klasioensis*, *M. longispina*, *M. manibuii*, *M. sembrae* and *M. susii* are genetically differentiated from each other and therefore constitute reproductively isolated entities.

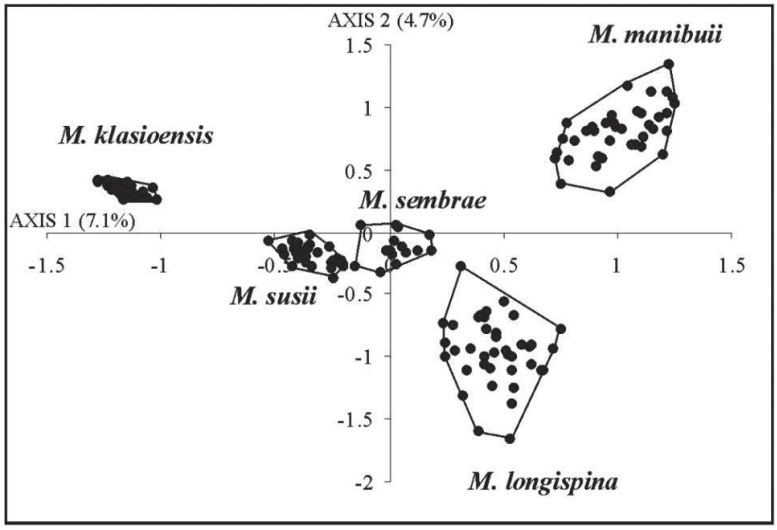


Figure 15. Factorial correspondence analysis of genotypes (164 individuals; 12 loci) from the five new species belonging to COI "Northern Bird's Head" clade. Projection on axis 1 and axis 2 segregating the five new species *M. klasioensis*, *M. longispina*, *M. manibuii*, *M. sembrae* and *M. susii*.

Tabel 13. Pairwise Fst values between species from the cluster "Central Ayamaru Plateau" and *M. arfakensis.*

	M. klasioensis	*M. ajamaruensis*	*M. fasinensis*	*M. susii*	*M. arfakensis*	*M. boesemani*	*M. ericrobertsi*
M. longispina	0.407	0.191	0.354	0.254	0.374	0.171	0.338
M. klasioensis	-	0.398	0.633	0.418	0.597	0.361	0.707
M. ajamaruensis	-	-	0.415	0.348	0.427	0.072	0.418
M. fasinensis	-	-	-	0.587	0.595	0.412	0.231
M. susii	-	-	-	-	0.512	0.322	0.601
M. arfakensis	-	-	-	-	-	0.432	0.647
M. boesemani	-	-	-	-	-	-	0.393

Tabel 14. Pairwise Fst values between species composing the cluster "Southern Ayamaru Plateau", all valid species from "Central Ayamaru plateau cluster and *M. arfakensis*

Fst	*M. ajamaruensis*	*M. arfakensis*	*M. boesemani*	*M. sembrae*	*M. fasinensis*	*M. ericrobertsi*
M. multiradiata	0.238	0.415	0.198	0.355	0.498	0.488
M. ajamaruensis	-	0.427	0.072	0.288	0.415	0.418
M. arfakensis	-	-	0.432	0.484	0.596	0.647
M. boesemani	-	-	-	0.262	0.412	0.393
M. sembrae	-	-	-	-	0.517	0.524
M. fasinensis	-	-	-	-	-	0.231

(5) The three new species *M. naramasae*, *M. rumberponensis* and *M. sikuensis* are genetically differentiated from each other, as well as from the remaining species *M. angfa* and *M. parva* that compose the "Bird's Neck" cluster (Figure 16). All pairwise Fst values showed highly significant genetic divergence ($p < 0.001$) among these species (Table 15). Pairwise Fst values between the 3 new species were comprised between 0.356 and 0.714. Interestingly, *M. angfa* and *M. parva*, the two species that shared the same COI haplotype, displayed a significant pairwise Fst value of 0.460. The locus Mb_tri1 revealed diagnostic for these two species, with a fixed allele (104) for *M. angfa* and two alleles (110 (75%) and 113 (25%)) for *M. parva*.

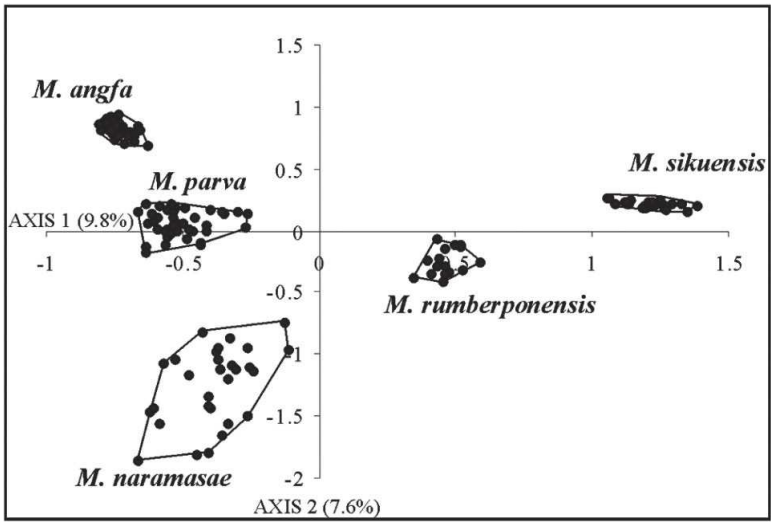


Figure 16. Factorial correspondence analysis of genotypes (164 individuals; 12 loci) from all the species belonging to cluster "Bird's Neck". Projection on axis 1 and axis 2 segregating the three new species *M. naramasae, M. rumberponensis*, and *M. sikuensis* with other species composing this cluster.

Tabel 15. Pairwise Fst values between species composing the cluster "Bird's Neck".

	M. parva	*M. angfa*	*M. rumberponensi*	*M. naramasae*
M. sikuensis	0.63	0.739	0.714	0.485
M. parva	-	0.46	0.545	0.245
M. angfa	-	-	0.707	0.401
M. rumberponensis	-	-	-	0.356

The microsatellite data therefore demonstrated that the eight new species represent distinct genetic entities, since they differ from each other and also from closely related valid species. Their species description are available in Nugraha *et al.,* 2015 (Annex 2). Because no intermediate genotypes and no overlap were observed between these taxonomic entities, we therefore considered that these species represent new species. The morphological description of the new species was not part of my work (Dr. Kadarusman PhD Thesis, 2012), they are not presented in my thesis but can be found in the corresponding published paper presented in Annex 2. The new species were given the names as listed in Figure 18

4.3.3. Mitochondrial DNA barcode and tree-based delimitation of West-Papuan species

The ML tree obtained from partial COI sequences obtained from 81 individuals belonging to 20 valid species and the 8 potential new species (Table 9) is presented in figure 24. The same topology was obtained for the K2P-NJ tree; thus, only bootstrap values were displayed together with those obtained for the ML tree.

Three major clades are highlighted on the ML tree (Figure 17). These results are congruent with the topology of the ML tree based on cytochrome b provided in Allen *et al.*, (2014 b). The first clade "Northern Bird's Head" (bootstrap: ML 96%, NJ 94%) includes all *Melanotaenia* species from the Bird's Head Peninsula (i.e. *M. ajamaruensis*, *M. arfakensis*, *M. boesemani*, *M. ericrobertsi*, *M. fasinensis*, *M. fredericki*, *M. multiradiata*) and from the Southern Raja Ampat Islands (i.e. *M. batanta*, *M. misoolensis*, *M. salawati*). It also includes 5 new species (i.e. *M. klasioensis*, *M. susi*, *M. longispina*, *M. sembrae*, *M. manibuii*). The second clade "Southern Bird's Head" (bootstrap: ML 98%, NJ 94%) includes all the *Melanotaenia* species from the Bomberai Peninsula (i.e. *M. ammeri*, *M. arguni*, *M. irianjaya*, *M. urisa*, *M. veoliae*, *M. wanoma*) and from the Bird's Neck (i.e. *M. angfa*, *M. parva*). It also includes 3 new species (i.e. *M.*

naramasae, *M. rumberponensis*, *M. sikuensis*). The third clade "Waigeo" is supported by high bootstrap values (ML 100%; NJ 99%) and includes the two species *M. catherinae* and *M. synergos* known from the Northern Raja Ampat (Figure 17).

The new species *M. manibuii* has a basal position in the first clade (Figure 17) and displays an important phylogenetic distance with all the species composing this clade (average K2P distance: 0.053 ± 0.008). *M. manibuii* and *M. irianjaya*, are morphologically closely related, although, they represent distinct species sorting in two different clades (i.e. frist clade for *M. manibuii* and second clade for *M. irianjaya*) and are separated by a high phylogenetic distances (K2P distance: 0.069 ± 0.009).

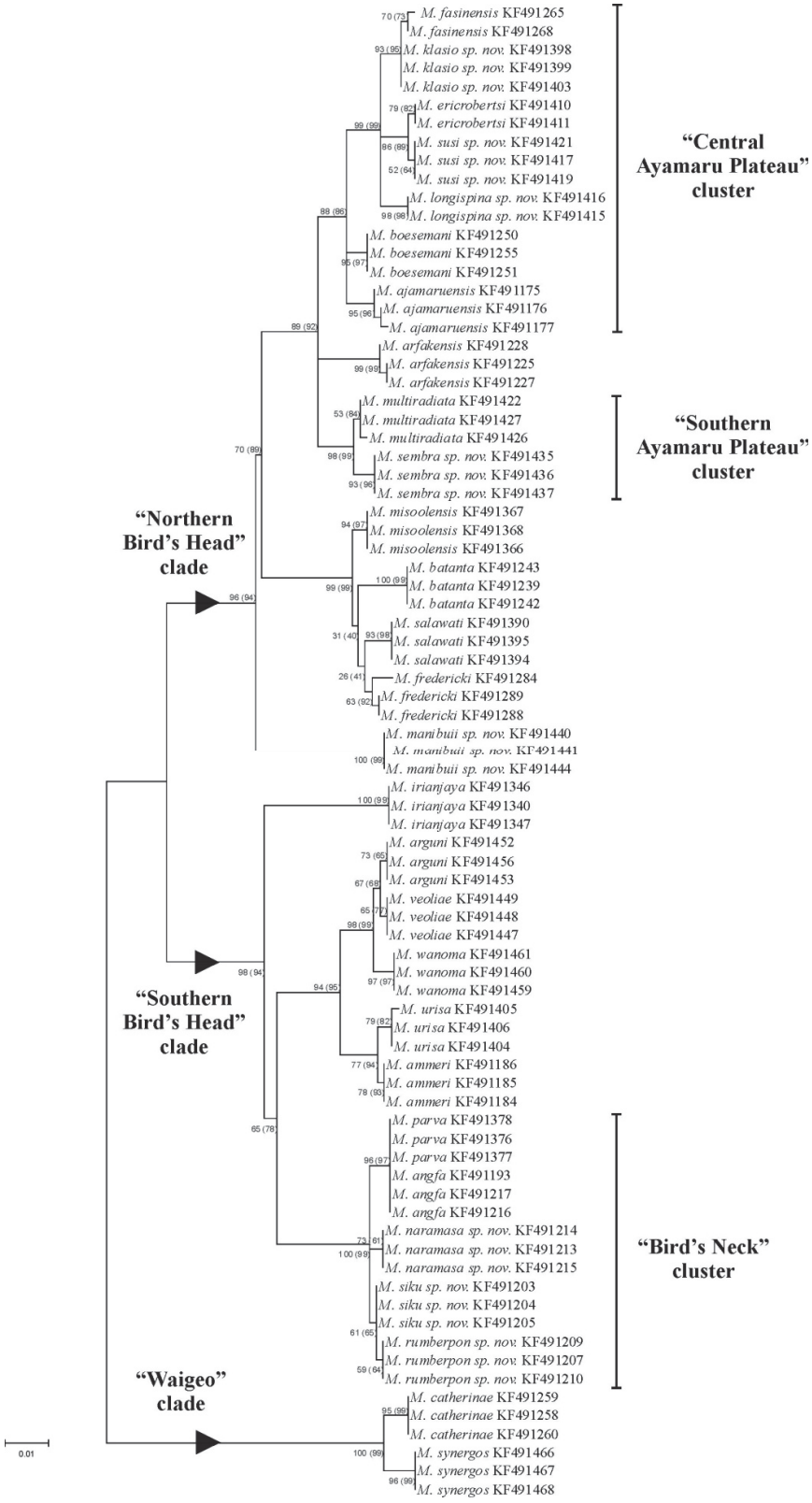


Figure 17. Maximum likelihood tree for Bird's Head region *Melanotaenia* species based on analysis of partial COI sequences (650 bp). Bootstrap values obtained for ML on 500 replicates and for NJ (within brackets).

The new species *M. sembrae* forms together with *M. multiradiata* the "Southern Ayamaru Plateau" cluster within the first clade (bootstrap: ML 98%, NJ 99%). The two species are characterized by low genetic divergence of less than 1% (K2P distance: 0.007 ± 0.003).

The new species *M. klasioensis*, *M. susii* and *M. longispina* belong to the cluster "Central Ayamaru Plateau" together with *M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi* and *M. fasinensis* (bootstrap: ML 88%, NJ 86%). These species are characterized by low phylogenetic distances (average K2P distance: 0.015 ± 0.003) and are all distributed in the fragmented river systems of the karstic Ayamaru Plateau. This cluster displays higher phylogenetic distances with *M. arfakensis* (K2P distance: 0.030 ± 0.005) as well as with the "Southern Ayamaru Plateau" cluster (K2P distance: 0.029 ± 0.005).

Finally, the 3 new species *M. naramasae*, *M. sikuensis* and *M. rumberponensis* are genetically closely related with *M. angfa* and *M. parva*. They belong to the cluster "Bird's Neck" and are characterised by phylogenetic distances of less than 1% (K2P distance: 0.005 ± 0.002). This cluster, however, displays a higher phylogenetic distances with the remaining species composing the "Southern Bird's Head" clade (K2P distance: 0.046 ± 0.007). Interestingly, the COI failed to differentiate *M. angfa* from *M. parva*, which display the same barcode sequence. This absence of genetic differentiation among these species was also revealed by Allen *et al.*, (2014 b) for the cytochrome b gene and by Unmack *et al.*, (2013) on one nuclear gene (S7) and seven mitochondrial markers (ND1, ND2, ND4L, ND4, ATPase6/8, cyt b, and partial COIII).

Figure 18. Description and corresponding picture of the eight new species of *Melanotaenia* from West Papua.

No	Species name	Picture
1	***Melanotaenia klasioensis*** Type locality of *Melanotaenia klasioensis, sp. nov,* Klasio Creek, near Malabolo, Sorong selatan, West Papua-Indonesia	
2.	***Melanotaenia longispina*** Type locality of *Melanotaenia longispina*, Klahfot River, near Saluk, Sorong Regency, West Papua - Indonesia	 
3.	***Melanotaenia susii*** Type locality of *Melanotaenia susii sp.nov*. Susi Creek, Sorong Selatan, West Papua – Indonesia	

4. ***Melanotaenia sembrae***

Only from the Sembra River.

5. ***Melanotaenia manibuii***

Only from the Tisbo River.

6. ***Melanotaenia naramasae***

Type locality of *Melanotaenia naramasa sp.nov.* Naramasa river, Bird's Neck, west Papua-Indonesia

7. ***Melanotaenia rumberpon*ensis**

Only from the Rumberpon Island.

8. ***Melanotaenia siku*ensis**

Only from the Siku Creek.

4.4. DISCUSSION

The only five species (*i.e. M. ammeri, M. fredericki, M. irianjaya, M. manibuii, M. naramasae*) presenting significant multilocus Hardy-Weinberg deviations consisted of a mix of individuals sampled at various locations along the same river drainage, because of an insufficient number of specimens that could be caught. These results suggest the possible occurrence of genetically differentiated sub populations patchily distributed along the same river and must be taken into consideration in future sampling strategies or population monitoring of *Melanotaenia* species. The majority of Hardy-Weinberg disequilibria reported for the different species were probably due to Wahlund effect, as reported by Johnson & Black (1984). The Wahlund effect refers to the reduction of heterozygosity in a population caused by a structuration into subpopulations. Namely, if two or more subpopulations have different allele frequencies, then the overall heterozygosity is reduced, even if the subpopulations themselves are under Hardy-Weinberg equilibrium.

The mitochondrial DNA phylogeny tree combined with the microsatellite genotyping revealed previously undetected diversity among West Papua rainbowfishes. However, the use of the 12 new microsatellite markers proved useful and allowed to confirm these taxa as new species. It revealed an unexpected species diversity of West Papuan Rainbowfishes. Prior to this work, 24 valid species had been described from the region. The discovery of 8 new

species in the area emphasized a cryptic diversity that was largely underestimated. Seven of these 8 newly discovered species are confined to a single river system localized in fragmented karstic environments such as in the limestone karst formations from Ayamaru Plateau or in the northern extension of Lengguru Range. *Melanotaenia manibuii*, which was discovered on the southern foothills of Arfak mountains, is the only species distributed outside a karstic formation.

Due to intense tectonic movements of uplift or subsidence and important erosion activity that happened during more than 10 million years, karst systems are characterized by endorheic drainage basins consisting of isolated lakes or fragmented rivers with aerial course which length varies from only several hundred meters to a few kilometers. These unique geological formations have therefore promoted intense diversification and numerous speciation events in Rainbowfishes from Western Papua (Kadarusman *et al.*, 2010, 2012b; Allen and Hadiaty, 2013; Allen *et al.*, 2014a, b). The total area covered during expedition field organized since 2007 represents approximately 20,000 km2, which corresponds to less than 10% of the global terrestrial surface of Western Papua and to less than 20% of karst landscapes. Therefore, there are still many unexplored areas that remain to be studied, with a special attention in the Central Bird's Head lowlands eastward of Ayamaru Plateau between southern Tambrauw and Arfak mountains and along the Northern foothills of Kumawa on Bomberai Peninsula.

The Lengguru fold-and-thrust Belt (LFTB) combined with the Central Dividing Range proved to have played a key role in the diversification of *Melanotaeniidae* at the regional scale (Kadarusman *et al.*, 2012a; Unmack *et al.*, 2013). Rapid tectonic uplift and subsidence raised in Mid-Miocene the radiation of 4 major monophyletic clades, each restricted to a specific biogeographic province with Western Papua Province probably being the center of origin of the genus *Melanotaenia*. The fact that all the species from Western Papua belonged to the same phylogenetic clade demonstrates their genetic relatedness and proves that dispersal with adjacent biogeographic provinces (*i.e.* Northern or Southern New Guinea) was interrupted since the geological formation of the LFTB. All the species of melanotaeniids from Western New Guinea are therefore endemic from

the area. Because they are all obligate freshwater fish, further investigations on the diversity of other obligate freshwater taxa of this region, such as *Hephaestus*, *Glossamia* and *Zenarchopterus*, appear very promising.

V. GENETIC DIVERSITY AND GEOGRAPIC ORIGIN OF *MELANOTAENIA BOESEMANI* REARED IN INDONESIAN FARMS

5. 1. INTRODUCTION

The Boeseman´s Rainbowfish *Melanotaenia boesemani* Allen & Cross, 1980 is one of the most popular rainbowfish species in the aquarium hobby. When fully matured, males display a very distinct pattern of coloration compared to other species with a half-and-half coloration marked by a brilliant blue anteriorly and by a bright yellow to orange-red posteriorly (Figure 19) (Kadarusman, 2012; Tappin, 2010).

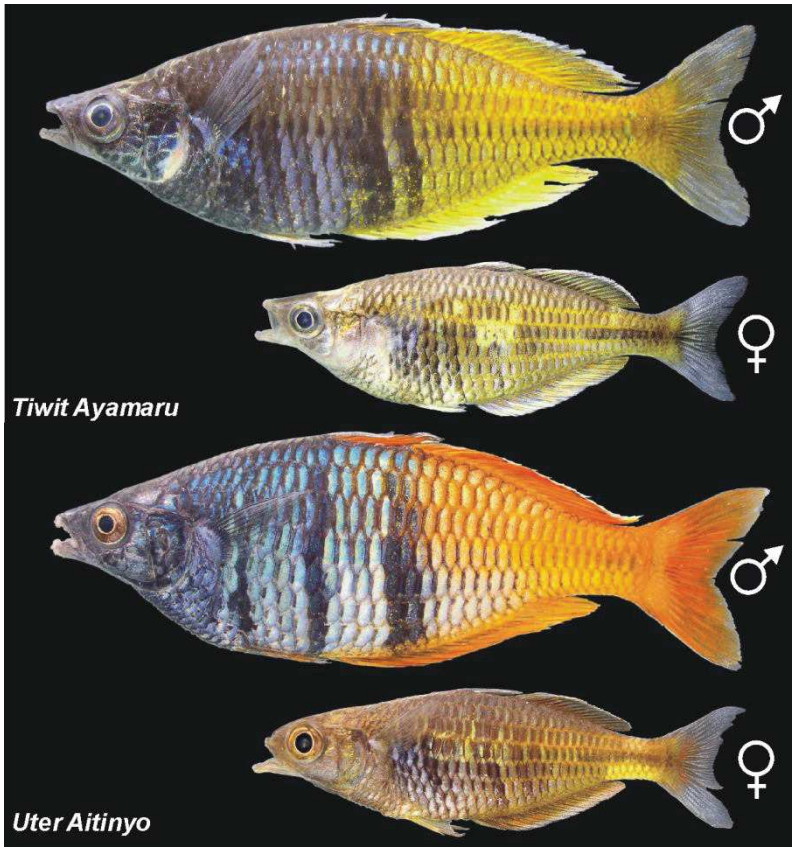


Figure 19. Coloration patterns of Boeseman´s Rainbowfish *Melanotaenia boesemani* from Ayamaru and from Uter lake (Photo Dr. Laurent Pouyaud).

Gerald Allen discovered the species while studying the material collected in 1954-55 by Marinus Boeseman and stored at the National Museum of Natural History in Leiden (Netherlands) (Allen, 1980). According to several studies (Allen, 1995; Allen, 1982; Kadarusman, 2012), the species is only known from Ayamaru Lakes and tributaries and also from Uter-Aitinyo Lake (or Uter Lake). The two locations are distant by 30 km and are separated by rugged karsts (Fig. 20).

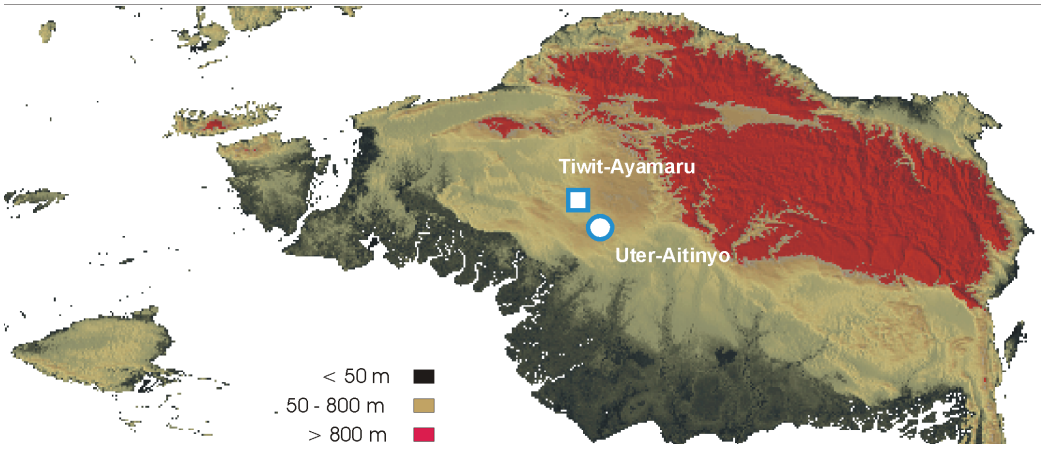


Figure 20. Geographic localization of Ayamaru and Uter Lakes in West Papua, Indonesia.

Although they show clear morphological distinctions (Figure 20), analysis of the populations from the two lakes with different molecular markers (cytochrome oxidase I, ribosomal protein S7 and cytochrome oxidase b) revealed that they are genetically very similar. After the first publication describing *M. boesemani* (Allen, 1980), a great interest arose for the potential commercial value of this species. It was introduced to the aquarium hobby in 1983 and has steadily increased in popularity since then (Polhemus, 2007). In the mid 1980s, more than 60,000 males were caught monthly from Ayamaru for exportation purposes (Smith *et al.*, 2007). This over-exploitation has therefore quickly conducted this species to the verge of extinction in its natural habitat (Allen, 1995). As a consequence, it has been placed on the red list of endangered species since 2004, and only aquaculture products are now supposed to be exported (IUCN, 2015).

Although there is a lack of precise data, the number of Indonesian farms breeding this species seems to be extremely low, with no more than 10. They export more than 1000 males per week, at price of about 0,75 - 2 US $ per individual, depending on its size, which is usually comprised between 1.75 and 2.5 inches (personal communication from the Jakarta fish quarantine). All the farms are located around Jakarta, and their production is not likely to account for the totality of exported fish. *Melanotaenia boesemani* has been domesticated and produced in Indonesian farms since 1983. At present, farmers claim a decrease of the production in terms of quantity and quality, *i.e.*: males are not as colored as in the wild; growth rate and fecundity are slower; the number of females per spawning is higher; many specimens display a different but typical morphology (which the producers call "perrot", figure 21).

Figure 21. *Melanotaenia boesemani* with a malformation morfologie and coloration.

They interpret these observations as a consequence of a loss of genetic variability and possible inbreeding (personal communication). The decline in fitness components with inbreeding has attracted the attention of geneticists and evolutionary biologists since Darwin. As was realized early after the rediscovery of Mendelian genetics, the deleterious effects of inbreeding reflect the consequences of increased homozygosity for alleles affecting fitness.

In this context, we implemented the new microsatellite markers in view of assessing the genetic variability of six different strains of *M. boesemani* reared in six Javanese farms, as well as to compare their genetic variability with that of

wild populations for determining the geographic origin of the founder breeders and for quantifying a possible loss of variability.

5. 2 MATERIAL AND METHODS

This research was held from December 2013 – August 2014, partly in Jakarta and its region (for fish sampling) and in the laboratory of the *'Institut des Sciences de l'Evolution de Montpellier, Université de Montpellier, France* (for genetic analyses)

5.2.1. Origin and sampling of the investigated fish

This research used 183 captive *M. boesemani* specimens from six local cultivation farms located around Jakarta in West Java (Table 16). Based on a preliminary survey realized in 9 farms, these 6 farms have been cultivating *M. boesemani* for many years (up to 30 years), do not practice out-crossings with other breeders and are facing difficulties such as a higher number of females in each harvest (while only males are suitable for sale), a smaller body size and a loss of color brightness. Approximately 30 individuals were collected from each farm. Fish were anaesthetized with 0.1 mL/L Eugenol and a ~1-cm^2 piece of anal fin was collected and stored in absolute ethanol for further DNA extractions. Fish were then allowed to recover from anesthetic and were released back into the ponds. In order to investigate the possible origin of these reared *M. boesemani* fish, we also analyzed two *M. boesemani* wild populations: one from the vicinity of Ayamaru lake (Tiwit tributary, 1°15.463'S and 132°14.939'E, 28 specimens) and one from Uter lake (1°25.957'S and 132°23.258'E, 49 specimens), which are the only known localities where this species can be found. These wild specimens were collected during the 2007 Lengguru expedition.

Table 16. List of *M. boesemani* farmers in Jakarta and Bekasi.

No	Farmers name and addres	Year Cultivation	Number of Samples
1	Farm Gusi, Ciracas	1983	34
2	Farm Sukri, Depok	2000	30
3	Farm Hasan, Bekasi	1983	30
4	Farm Yahya, Bekasi	2009	30
5	Farm Didi, Tridayasakti, Bekasi	1986	29
6	Farm Warso, Tambun Utara, Bekasi	2012	30

5.2.2. DNA extraction and microsatellite amplification

Individual samples were genotyped with the 12 nuclear microsatellite markers developed in chapter III. DNA was extracted from a small fragment (10 mg) of anal fin clip with the NucleoSpin® 96 Tissue kit (Macherey-Nagel), according to the manufacturer's instructions, using a Janus automated Workstation (Perkin Elmer). Forward primers were end-labeled with fluorescent dyes (5'FAM, 5'HEX, 5'ATO550, 5'ATO565) (Eurofins) and each reaction contained 5 µl of 2x Master mix (Fast-Start PCR kit, Roche), 0.1 µM of forward primer, 0.4 µM of reverse primer and 0.5 µl of template DNA. Cycling conditions consisted in an initial denaturation at 95°C for 4 min followed by 30 cycles of 95°C for 20 s, 56°C for 20 s and 72 °C for 30 s, and a final elongation step of 7 min at 72°C. Amplicon size was analyzed by capillary electrophoresis as described in the previous chapters, in the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier). Allele sizing and genotyping were achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems).

5.2.3. Genetic diversity analysis

Allelic numbers (Na), average observed (Ho) and expected (He) heterozygosities were calculated using the GENETIX 4.05 software (Belkhir, 1996). Data were analyzed for possible departures from Hardy-Weinberg equilibrium by estimating the significance of the multilocus inbreeding coefficient

(Fis) from sets of 1000 random allelic permutations of the original datasets. The genetic structure among the investigated populations was also evaluated by calculating pairwise Fst values with 1000 random allelic permutations. The significance level of *P* value for Fis and Fst was defined as the probability to obtaining absolute values higher than or equal to the observed one under the null hypothesis. The geographic origin of the 6 reared strains was assessed through a factorial analysis of correspondences (Genetix 4.05), by comparing their genotype frequencies to those of the wild populations of *M. boesemani* from Ayamaru and Uter lakes.

5.3. RESULTS

5.3.1. Genetic variability and heterozygosity of *M. boesemani* in aquaculture settings and in natural populations

A total of 151 alleles were detected in the 183 farmed and 77 wild specimens across the 12 microsatellite loci. The total number of alleles per locus for all populations varied from 5 to 22 and all loci were polymorphic in each strain and wild population (Table 17). Generally, the domesticated populations showed a lower genetic variability (total number of alleles comprised between 62 and 86) compared to Ayamaru wild population (107 alleles), but comparable with that observed in the wild population of Uter (82 alleles) (Table 17). Considering only the wild populations, there were 23 private alleles for Uter Lake population and 48 private alleles for Ayamaru Lake. When all samples were included, there were 17 private alleles for Uter Lake, 16 private alleles for Ayamaru Lake and 21 private alleles for the domesticated populations (*i.e.* only present in one or more strains and absent in wild populations). There were 6 alleles shared between Uter Lake and one or more strains, and 32 alleles shared between Ayamaru lake and at least one strain. There were 51 alleles shared between both wild populations and one or more strains, and 8 alleles shared between Ayamaru and Uter lakes but absent from any strains (Table 20). Likewise, the heterozygosities calculated for the captive populations (Hobs comprised between 0.53 and 0.64) were lower than

that calculated for the Ayamaru wild population (0.68) but similar to that of Uter population (0.56) (Table 18). Moreover, the Ho were never significantly different from the He. The multilocus Fis values for all populations (wild and captive) were comprised between -0.010 and 0.061 and none of them was significant except that obtained for the Sukri strain (Fis = 0.061, $P < 0.05$) (Table 18). These results strongly suggest that, with the exception of Sukri farm, there is no deficit in heterozygotes in any population. Finally, the pairwise Fst values calculated between each population were all significant ($P < 0.05$), except between Warso and Yahya and between Warso and Didi farms, indicating that most of the populations are genetically differentiated from each other (Table 19).

Table 17. Number of alleles per locus for each population.

Locus	Uter Lake n=49	Ayamaru Lake n=28	Gusi farm n=34	Sukri farm n=30	Hasan farm n=30	Yahya farm n=30	Didi farm n=29	Warso farm n=30
Mb_di1	9	14	8	8	10	9	9	8
Mb_di2	13	14	9	8	10	9	9	9
Mb_di3	10	8	7	5	7	7	5	6
Mb_penta1	6	8	8	7	7	7	6	6
Mb_di4	10	15	10	8	11	12	7	13
Mb_tetra1	6	7	3	4	6	5	2	3
Mb_tri1	4	4	4	3	3	2	2	2
Mb_tri2	3	4	4	4	4	5	3	4
Mb_di5	6	8	8	9	8	7	6	7
Mb_tetra2	10	18	10	11	12	6	6	7
Mb_tri3	3	3	3	3	4	2	3	2
Mb_tri4	2	4	5	4	4	4	4	4
TNA*	82	107	79	74	86	75	62	71

*TNA: total number of alleles

Table 18. Sample size (N), average observed (Ho) and expected (He) heterozygosity, and multilocus Fis values for each wild population and strain.

Population	N	Hobs	Hexp	Fis
Uter lake	49	0.56	0.55	-0.010
Ayamaru lake	28	0.68	0.69	0.013
Farm Gusi	34	0.61	0.61	-0.007
Farm Sukri	30	0.59	0.63	0.061*
Farm Hasan	30	0.64	0.64	-0.001
Farm Yahya	30	0.61	0.62	0.010
Farm Didi	29	0.53	0.55	0.029
Farm warso	30	0.62	0.61	-0.013

* P < 0.01

Table 19. Population differentiation based on pairwise Fst estimates.

	Ayamaru Lake	Gusi	Sukri	Hasan	Yahya	Didi	Warso
Uter Lake	0.176***	0.228***	0.226***	0.221***	0.249***	0.263***	0.231***
Ayamaru Lake		0.030***	0.028***	0.028***	0.062***	0.075***	0.054***
Gusi			0.012***	0.032***	0.057***	0.055***	0.053***
Sukri				0.015**	0.029***	0.038***	0.032***
Hasan					0.030***	0.036***	0.028***
Yahya						0.011*	0.002 NS
Didi							0.007 NS

NS: non significant; * p<0.05; ** p<0.01; *** p<0.001

Table 20. Allelic frequency for each population.

Allele size at each locus (nt)	Uter Lake	Ayamaru lake	Gusi farm	Sukri farm	Hasan farm	Yahya farm	Didi farm	Warso farm
Mb_di1								
87	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
89	0.020	0.036	0.000	0.000	0.000	0.000	0.000	0.000
95	0.357	0.125	0.206	0.183	0.117	0.067	0.017	0.050
97	0.061	0.018	0.044	0.083	0.017	0.000	0.000	0.000
101	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
103	0.000	0.000	0.147	0.050	0.100	0.033	0.000	0.017
105	0.000	0.036	0.000	0.000	0.017	0.000	0.052	0.000
107	0.000	0.089	0.000	0.000	0.000	0.000	0.000	0.000
109	0.378	0.018	0.088	0.067	0.067	0.150	0.103	0.233
111	0.031	0.000	0.000	0.000	0.000	0.033	0.017	0.050
113	0.071	0.250	0.235	0.450	0.433	0.500	0.448	0.367
115	0.010	0.036	0.029	0.000	0.000	0.050	0.069	0.000
117	0.061	0.304	0.221	0.117	0.100	0.050	0.121	0.083

119	0.000	0.018	0.029	0.033	0.117	0.100	0.138	0.133
121	0.000	0.000	0.000	0.017	0.017	0.017	0.000	0.067
125	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
127	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
137	0.000	0.000	0.000	0.000	0.017	0.000	0.035	0.000
139	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
Mb_di2								
113	0.000	0.089	0.029	0.033	0.167	0.033	0.121	0.033
117	0.083	0.000	0.029	0.083	0.067	0.000	0.017	0.000
119	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
123	0.000	0.054	0.059	0.000	0.050	0.000	0.017	0.017
125	0.031	0.018	0.000	0.000	0.000	0.000	0.000	0.000
127	0.021	0.036	0.044	0.083	0.017	0.000	0.000	0.000
129	0.260	0.036	0.029	0.150	0.067	0.083	0.103	0.067
131	0.042	0.054	0.000	0.000	0.017	0.100	0.000	0.133
133	0.010	0.071	0.147	0.100	0.067	0.083	0.052	0.033
135	0.031	0.268	0.279	0.250	0.200	0.300	0.172	0.217
137	0.333	0.071	0.000	0.000	0.167	0.167	0.121	0.217
139	0.115	0.161	0.279	0.267	0.183	0.167	0.345	0.250
141	0.021	0.054	0.103	0.033	0.000	0.033	0.052	0.033
143	0.021	0.036	0.000	0.000	0.000	0.000	0.000	0.000
145	0.000	0.036	0.000	0.000	0.000	0.033	0.000	0.000
147	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000
149	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mb-di3								
145	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000
153	0.160	0.107	0.061	0.167	0.133	0.100	0.086	0.117
155	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000
157	0.000	0.000	0.015	0.033	0.050	0.017	0.000	0.033
159	0.000	0.107	0.000	0.000	0.000	0.017	0.000	0.000
161	0.000	0.179	0.015	0.000	0.017	0.033	0.035	0.017
163	0.000	0.446	0.788	0.650	0.650	0.550	0.603	0.433
165	0.021	0.071	0.030	0.000	0.033	0.033	0.017	0.067
167	0.000	0.018	0.000	0.033	0.033	0.000	0.000	0.000
169	0.011	0.054	0.000	0.000	0.000	0.000	0.000	0.000
171	0.000	0.000	0.076	0.117	0.083	0.250	0.259	0.333
173	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
175	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000
179	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
181	0.521	0.000	0.000	0.000	0.000	0.000	0.000	0.000
183	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000
185	0.096	0.000	0.000	0.000	0.000	0.000	0.000	0.000
187	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Mb_Penta 1								
160	0.644	0.268	0.368	0.167	0.133	0.017	0.018	0.000
165	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
170	0.000	0.054	0.000	0.000	0.000	0.000	0.000	0.000
175	0.000	0.000	0.147	0.067	0.083	0.067	0.036	0.069
180	0.000	0.071	0.074	0.100	0.167	0.283	0.286	0.138
185	0.011	0.214	0.044	0.083	0.217	0.217	0.232	0.259
190	0.078	0.232	0.191	0.267	0.200	0.017	0.036	0.138
195	0.167	0.125	0.029	0.033	0.133	0.067	0.000	0.017
200	0.033	0.018	0.132	0.283	0.067	0.333	0.393	0.379
205	0.067	0.000	0.015	0.000	0.000	0.000	0.000	0.000
Mb_di4								
98	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000
100	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
106	0.020	0.000	0.029	0.000	0.000	0.000	0.000	0.000
110	0.000	0.000	0.074	0.000	0.000	0.000	0.000	0.017
112	0.296	0.268	0.324	0.483	0.150	0.433	0.328	0.283
114	0.378	0.143	0.147	0.083	0.283	0.117	0.103	0.150
116	0.051	0.036	0.000	0.000	0.000	0.017	0.000	0.017
118	0.031	0.089	0.044	0.033	0.017	0.017	0.000	0.117
122	0.010	0.000	0.000	0.000	0.017	0.017	0.000	0.000
124	0.143	0.143	0.132	0.217	0.283	0.200	0.276	0.217
126	0.031	0.036	0.015	0.017	0.000	0.050	0.172	0.083
128	0.031	0.054	0.000	0.000	0.017	0.000	0.000	0.000
130	0.000	0.054	0.000	0.000	0.000	0.000	0.000	0.000
132	0.000	0.018	0.000	0.000	0.017	0.067	0.069	0.033
134	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.017
138	0.000	0.018	0.000	0.017	0.000	0.000	0.000	0.000
140	0.000	0.000	0.162	0.033	0.033	0.017	0.000	0.017
142	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
144	0.000	0.018	0.000	0.000	0.067	0.000	0.017	0.000
148	0.000	0.018	0.000	0.000	0.100	0.033	0.000	0.017
152	0.000	0.036	0.000	0.000	0.000	0.017	0.000	0.017
164	0.000	0.000	0.059	0.117	0.017	0.017	0.035	0.017
Mb_Tetra 1								
131	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
135	0.010	0.036	0.000	0.000	0.017	0.000	0.000	0.000
137	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000
139	0.184	0.036	0.000	0.000	0.000	0.000	0.000	0.000
143	0.735	0.607	0.779	0.717	0.741	0.767	0.931	0.833
147	0.031	0.125	0.177	0.217	0.052	0.150	0.069	0.117
149	0.000	0.071	0.044	0.000	0.000	0.000	0.000	0.000
151	0.020	0.018	0.000	0.000	0.035	0.033	0.000	0.000

157	0.000	0.107	0.000	0.050	0.121	0.033	0.000	0.050
169	0.000	0.000	0.000	0.017	0.000	0.017	0.000	0.000
Mb_Tri 1								
107	0.000	0.018	0.044	0.000	0.000	0.000	0.000	0.000
113	0.000	0.839	0.882	0.900	0.931	0.817	0.948	0.862
116	0.702	0.071	0.015	0.017	0.052	0.000	0.000	0.000
119	0.043	0.071	0.059	0.083	0.017	0.183	0.052	0.138
122	0.096	0.000	0.000	0.000	0.000	0.000	0.000	0.000
125	0.160	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mb_Tri2								
138	0.000	0.143	0.206	0.283	0.300	0.383	0.207	0.333
144	0.958	0.554	0.662	0.533	0.583	0.483	0.776	0.617
145	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
147	0.000	0.286	0.118	0.167	0.067	0.083	0.017	0.017
150	0.031	0.018	0.015	0.017	0.050	0.033	0.000	0.033
153	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000
Mb_di5								
83	0.000	0.000	0.059	0.167	0.250	0.172	0.103	0.100
85	0.010	0.018	0.162	0.017	0.000	0.000	0.000	0.000
87	0.000	0.071	0.000	0.000	0.067	0.052	0.069	0.067
89	0.000	0.089	0.000	0.033	0.033	0.000	0.000	0.000
91	0.500	0.446	0.427	0.433	0.317	0.328	0.431	0.417
93	0.000	0.143	0.000	0.083	0.067	0.035	0.000	0.017
95	0.398	0.161	0.059	0.067	0.200	0.259	0.224	0.200
97	0.010	0.054	0.088	0.067	0.050	0.121	0.069	0.167
99	0.051	0.018	0.000	0.000	0.000	0.000	0.000	0.000
101	0.031	0.000	0.132	0.017	0.017	0.035	0.103	0.033
105	0.000	0.000	0.059	0.117	0.000	0.000	0.000	0.000
113	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000
Mb_Tetra2								
156	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
160	0.051	0.000	0.000	0.000	0.000	0.000	0.000	0.000
168	0.000	0.000	0.044	0.017	0.033	0.000	0.000	0.052
172	0.010	0.054	0.000	0.000	0.000	0.000	0.000	0.000
176	0.010	0.036	0.000	0.000	0.000	0.000	0.000	0.000
180	0.020	0.196	0.338	0.207	0.200	0.133	0.155	0.241
184	0.214	0.054	0.118	0.017	0.017	0.000	0.000	0.000
188	0.133	0.089	0.088	0.086	0.050	0.000	0.000	0.000
192	0.388	0.161	0.015	0.052	0.017	0.000	0.000	0.000
196	0.092	0.071	0.044	0.017	0.017	0.067	0.000	0.000
200	0.071	0.018	0.029	0.035	0.050	0.033	0.017	0.017
204	0.010	0.071	0.162	0.310	0.300	0.283	0.379	0.293
208	0.000	0.071	0.000	0.121	0.217	0.383	0.259	0.276

212	0.000	0.036	0.132	0.121	0.033	0.100	0.172	0.035
220	0.000	0.036	0.000	0.000	0.017	0.000	0.000	0.000
224	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
228	0.000	0.018	0.000	0.017	0.050	0.000	0.017	0.086
232	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
240	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
248	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
252	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
Mb_Tri 3								
127	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000
133	0.684	0.857	0.794	0.767	0.776	0.867	0.857	0.900
142	0.306	0.089	0.162	0.183	0.121	0.133	0.107	0.100
148	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
154	0.000	0.054	0.044	0.050	0.069	0.000	0.036	0.000
Mb_Tri 4								
99	0.796	0.446	0.221	0.317	0.283	0.133	0.138	0.233
102	0.204	0.214	0.368	0.250	0.250	0.600	0.603	0.550
108	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
111	0.000	0.268	0.294	0.367	0.400	0.217	0.241	0.183
114	0.000	0.071	0.088	0.067	0.067	0.050	0.017	0.033

5.3.2. Geographic origin of M. boesemani reared in Indonesian farms

A factorial correspondence analysis (FCA) was performed on all multilocus genotypes obtained for the 6 captive and the 2 wild populations (Figure 22). The projection made on axis 1 (5.99%) and axis 2 (2.47%) indicated that the wild population from Uter Lake could be genetically differentiated from that of Ayamaru Lake. Moreover, all the fish collected from the farms (*i.e.* polygon "pooled strains") were partly grouped with those of Ayamaru Lake (*i.e.* polygon "Ayamaru lake"). The third axis of the FCA did not enable to separate the farm samples from Ayamaru wild population. Likewise, when Uter population was removed, the Ayamaru and farm specimens still overlapped. These results suggest that all captive fish originate from the region of Ayamaru Lake.

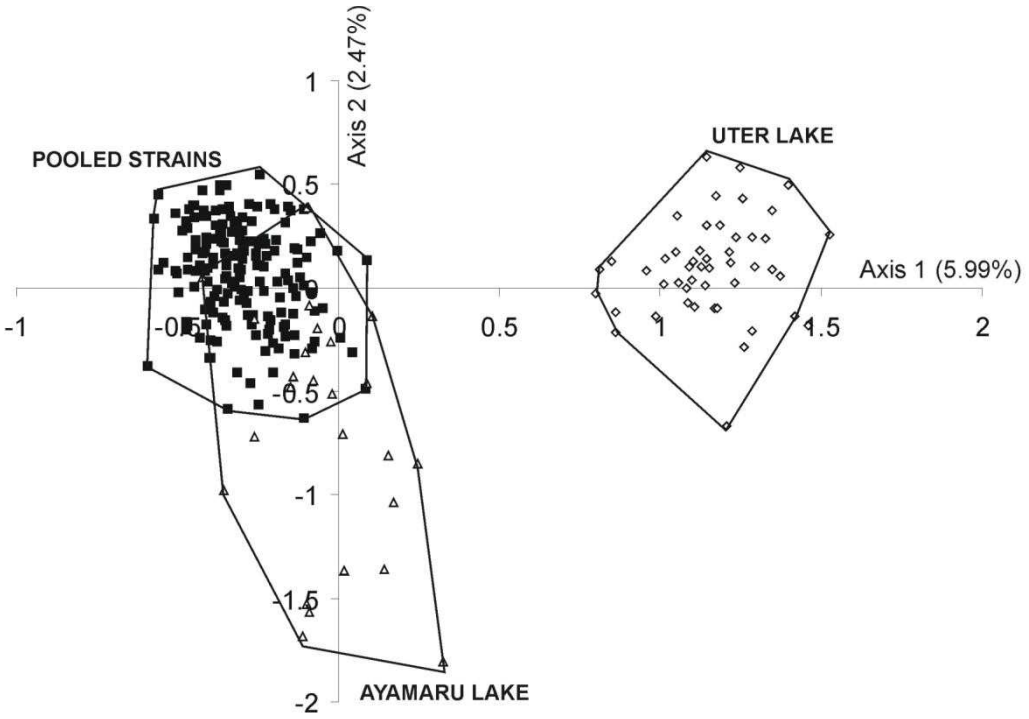


Figure 22. Factorial Component Analysis based on the multilocus genotypes of Boeseman's Rainbowfish *Melanotaenia boesemani* individuals from the 2 wild populations of Ayamaru (n=28) and Uter (n=49) and the 6 Indonesian farms (n=29-34).

5.4 DISCUSSION

Ayamaru and Uter lakes are located in Indonesia in the western part of New Guinea island. These two lakes are not connected and are separated by rugged karsts, with a distance of 30km. Though they are considered to belong to the same species, the fish from these two lakes display significant morphological differences: those from Ayamaru lake harbor a bright blue anterior and bright yellow anterior coloration, whereas those from Uter show a bright blue anterior and a reddish posterior coloration (Figure 19). Nevertheless, previous analyses based on both the mitochondrial cytochrome oxydase I gene and morphomeristic traits failed to separate these two populations, which are slightly overlapping (Kadarusman, 2012). Here, the use of 12 microsatellite markers brought clear evidence that they are genetically differentiated, and emphasizes that population mixtures should be strictly avoided during any upcoming broodstock constitution. From the samples investigated here, many private alleles were evidenced between the two wild populations of *M. boesemani* (23 for Uter lake and 48 for Ayamaru lake). Among the 12 markers, Mb_di1, Mb_di3, Mb_di4 and Mb_tetra2 accounted for more than 50% of the private alleles found between the two populations (42 over 71).

Among the investigated farms, Gusi, Hasan and Didi started to exploit *M. boesemani* in 1983 and 1986, respectively, *i.e*. quickly after the description of *M. boesemani* species by Allen and Cross (Allen, 1980) and the publication of *Rainbowfishes of Australia and Papua New Guinea* (Allen, 1982). This book greatly contributed to increase the popularity of rainbowfishes, especially the newly discovered New Guinea species, among which *M. boesemani* (Tappin, 2010). In spite of the establishment of these farms, exploitation of this species from its natural environment in Ayamaru has persisted until now. The geography of Uter Lake, surrounded by mountains and characterized by steep and rocky shores, is less favorable to fishing activities than that of Ayamaru, more easily accessible. This is probably the main reason why all investigated domesticated populations originated from Ayamaru.

The core population of Ayamaru sampled 30 years ago for the constitution of the cultivated strains was genetically different from that of the wild population sampled in 2007. This was especially evidenced by the 21 private alleles observed in the strains and absent in the wild population of Ayamaru Lake. The presence of these private alleles may suggest a loss of variability that the wild population of Ayamaru has undergone over the last 30 years. However, the fact that heterozygosity and allele number values calculated from the Ayamaru population are comparable with those of other rainbowfish species (Nugraha *et al*., 2014), is not in favor of a loss of genetic diversity. Most likely, these private alleles may indicate that the genetic diversity of the Ayamaru specimens investigated here are probably not representative of the whole genetic diversity of *M. boesemani* in this area (*i.e.* Ayamaru Lake and tributaries). The wild fish sampled in 2007 were indeed collected in Tiwit River, a tributary of Ayamaru Lake, and could represent a distinct genetic entity from the whole population. In this case, the species could constitute a metapopulation composed of several population subdivisions according to their geographic distribution in the mosaic of habitats characterizing Ayamaru Lake. It was recently shown on a wide range of marine fishes that overfishing could result in a significant reduction of genetic diversity (Pinsky *et al.*, 2014). Another recent meta-population study using many marine fish species demonstrated that the renewal of a fish population depends much less on the amount of available genitors than on environmental factors (Cury *et al*., 2014). If the data presented here is not sufficient to tackle these questions, it nonetheless highlights the urgent need to perform a complete and exhaustive sampling campaign for assessing the exact genetic structure and diversity of this endangered species, in order to efficiently contribute to its conservation. This implies to collect fish in many more locations of Ayamaru Lake and its tributaries. Indeed, in addition to overfishing, Ayamaru Lake also experiences important environmental threats (deforestation, urbanization) and episodic droughts. Based on the sequence of a mitochondrial DNA fragment of the native Australian freshwater fish *Rhadinocentrus ornatus*, Mather *et al*. showed that habitat degradation caused by urbanization entailed a significant reduction in genetic diversity (Mather *et al.*, 2015).

Finally, the genetic variability assessed within the 6 farmed populations was comparable to that of the natural population of Uter Lake. Besides, no deficit in heterozygotes was evidenced, suggesting that there was no major inbreeding in these reared populations. Therefore, the problems experienced by the farmers (*i.e.* decline of production, higher proportion of females per spawning, loss of coloration, lower growth rate and fecundity, morphological abnormalities) are obviously not due to inbreeding depression and are probably caused by other factors such as unsuitable management and/or poor water quality. It is noteworthy that all investigated farms are located in industrial and densely populated peri-urban areas. Considering that Jakarta and its suburbs have no waste water treatment system, the water used for rearing these fish is probably of very poor quality, and may contain many chemical and hormonal pollutants, which have both been proven to alter major fish traits such as reproduction, growth. It has indeed been shown that exposure to low concentrations of endocrine disruptive chemicals (among which estrogens) can impede gonadal function, reduce fertilization success, decrease fecundity, alter mating behavior and reverse sex of various aquatic species (Martinović *et al*., 2007; Sumpter, 2005 ;Vos *et al*., 2000). It can even cause the collapse of fish populations at trace concentrations (Kidd *et al*., 2007).

Regarding the coloration pattern, it is known that rainbowfish are able to change color according to the turbidity level of their environment (Kelley *et al.,* 2012; Rodgers *et al*., 2010). For instance, an increased brightness of red colors in environments rich in organic matter (*i.e.* more turbid) may help enhance conspicuousness, and then allow individuals to maintain communication in altered visual environments. Thus, the alteration of fish color brightness observed in reared animals may be the consequence of an adaptation to the aquaculture setting conditions, where ponds are very shallow and mimic habitats with full-spectrum lighting. However, a better understanding of the visual system of *Melanotaeniidae* is required to better predict how changes in the aquatic light environment affect the physiology and ecology of these fishes, and in turn, to allow farmers to adapt their rearing conditions in order to maintain bright body colors.

The combination of important levels of biodiversity with low human population density (2-6 inhabitants / km^2) led Conservation International to declare in 1997 New Guinea as the only "*Major Tropical Wilderness Area*" remaining in Asia. For illustration, all the species of melanotaeniids from Western New Guinea are endemic from the area. Because many rainbowfish species have restricted distributions and are confined to specific habitats, such as isolated lacustrine environments or small parts of a single river system, they are highly vulnerable to environmental disturbance and over-harvesting. Ayamaru Lake is affected by the development of residential areas and ecotourism. As a result, forest clearance has increased channel obstructions by sediments and led to partial drying of the lake. Meanwhile, simultaneous over-catching of the endemic *M. boesemani* conducted this species to the verge of extinction (Allen, 2007). The results presented here (*i.e.* no significant loss of heterozygotes in the wild populations and no inbreeding depression in the reared strains) indicate that, in spite of the threats that Ayamaru is facing, it is still possible to prevent the extinction of *M. boesemani*. This, however, implies to increase its aquaculture production in order to quickly alleviate the overfishing pressure. This, in turn, involves a better management of the quality of waters used for rearing *M. boesemani*, which is a general concern in Indonesia.

VI. GENERAL DISCUSSION AND PERSPECTIVES

Indonesia is one of the countries that hold the highest biodiversity in the world (Fretes 2007). New Guinea Island is located in the northern quadrant of the Australian tectonic plate. Papua has more than a thousand fringing islands, from tiny to quite large ones. The temperature gradient due to altitude elevation in mountainous Papua has contributed distinct biotas to inhabit adjacent patches of land, separated only by elevation. Together with the geology of this island, it certainly explains much of the species richness of Papua.

Polhemus and Allen (2007) defined 29 areas of freshwater endemism within New Guinea and closely adjacent islands that need further assessment for conservation strategies. These hypotheses of regional endemism were further refined for western New Guinea, especially for freshwater fish and and more widely for aquatic organisms for the whole New Guinea. These authors also identified the Bird's Head Peninsula as an independent biogeographic region characterized by important endemism rates. Beside most vertebrate groups, the Papuan freshwater fish fauna displays strong endemic rates including Rainbowfishes. For illustration, around 84% of the freshwater species are exclusively found in Papua island. Rainbowfish of the family Melanotaeniidae are the leading family of Papuan endemic species, especially in western New Guinea.

Conservation biology was developed to understand the processes influencing extinction. Genetics has been an important focus of conservation biology because it helps determine the evolutionary context of endangered species and enables the development of better management strategies. Genetic variation interpreted in a population genetics context can be used to reconstruct the evolutionary history, examine the contemporary status, and predict the future of endangered species. Model organisms, habitat and ecosystem have sometimes been used to examine the bases of conservation management approaches. Overall, the framework of evolutionary genetics theory furnishes an elegant approach to interpret the measured amounts of genetic variation and predict the future effects of evolutionary factors and management strategies for endangered species.

Conservation International Rapid Assessment Program (RAP) was created in 1990 to quickly provide biological information needed to catalyze conservation action and improve biodiversity protection. Papua is an important area for global conservation, because of its extraordinary biodiversity, unique ecosystem and many endemism and unique species / biotas. The combination of important levels of biodiversity with low human population density (2-6 inhabitants / km^2) led Conservation International to declare in 1997 New Guinea as the only "*Major Tropical Wilderness Area*" remaining in Asia. For illustration, all the species of melanotaeniids from Western New Guinea are endemic from the area. Because many rainbowfish species have restricted distributions and are confined to specific habitats, such as isolated lacustrine environments or small parts of a single river system, they are highly vulnerable to environmental disturbance and over-harvesting. For instance, Ayamaru Lake is affected by the development of residential areas and ecotourism. As a result, forest clearance has increased channel obstructions by sediments and led to partial drying of the lake. Meanwhile, simultaneous over-catching of the endemic *M. boesemani* conducted this species to the verge of extinction (Allen, 2007). The results presented in this manuscript (*i.e.* no significant loss of heterozygotes in the wild populations and no inbreeding depression in the reared strains) indicate that, in spite of the threats that Ayamaru Lake is facing, it is still possible to prevent the extinction of *M. boesemani*.

Our results also revealed an unexpected species diversity of West Papuan Rainbowfishes. Prior to this work, 24 nominal species were known from West Papua. The discovery of 8 new species in the area emphasized a cryptic diversity largely underestimated. Seven of these 8 newly discovered species were confined to a single river system localized in fragmented karstic environment. This, again, highlights the high vulnerability of these populations, and illustrates the need to protect West Papua. For the past half century, it has been widely recognized that the rate of species extinction was increasing and that many other species were in imminent danger of extinction. The major factors related to these extinctions and declines were over-harvesting from hunting, fishing, trapping, and other killing; loss, degradation, and fragmentation of habitat; and introduction of non-native

species such as pathogens, parasites, predators, and competitors (Diamond 1989). This is typically what West Papua is facing today.

Considering the many endemic species in Papua, there is an urgent need to conduct floral and faunal surveys throughout Papua to identify areas of special conservation value. Many freshwater fish, particularly the rainbowfish (*Melanotaeniidae*), appear to be restricted to an isolated lake or small part of single river system. These species are highly vulnerable to environmental disturbances such as clear-cut logging, mining and building construction. For environment and biodiversity preservation, the government must increase its conservation policies. This implies that local populations be aware of the uniqueness of their plants and animals, and of the need to protect them. This implementation is of course difficult, especially in a context of economic development. However, this is part of our responsibilities to make citizens understand that biodiversity is important to people because it supports their livelihoods and welfare either directly through the use of biological resources, or indirectly through its contribution towards the maintenance of ecosystem function. Land with high biodiversity like Papua is able to provide services like watershed protection and climate regulation, which help maintain this biodiversity at a broader level. Biodiversity has to be considered at three levels, i.e. genetic, organism and ecosystem.

Such conservation plans must result from collaborations between local governments and several ministries such as the Ministry of Marine Affairs and Fisheries, the Ministry of Forestry, the Ministry of Agriculture and the Ministry of Environment. Indonesian government has already promulgated several laws for conservation management of living resources and their ecosystem, but more efforts are still necessary in order to preserve this area. Indonesian regulation for species protection is now compatible with the rules of IUCN. Until now, 70 nature conservation areas currently exist in Papua, while only 29 have been granted legal status. According to Frettes (2007), 16 endemic species of rainbowfish are covered under protected areas, corresponding to a geographic coverage of only about 27% of their entire habitat. If Papua has recently attracted much

conservation interest and investments from conservation organization, still a lot remains to do for protecting its wilderness.

The primary aim of conservation is to save the species in their natural environment. If the importance of conserving the wild ancestors and related species both *in situ* and *ex situ* is widely recognized, domestication is however often seen as the first (and even the only) way of conservation. The purpose of reorganization of wild populations into domestic and cultivated forms varies according to the interests of people: conserve genetic resources and/or use them in breeding programs. A problem resulting from domestication is the possibility of significant reduction of genetic variability, as has been observed in most species with advanced level of domestication (Arnaiz *et al*. 2003). In Indonesia, the aquaculture of ornamental fish has become an important industry. However, farming of the endangered *Melanotaenia boesemani* has remained anecdotic, which is reflected by the low number of farms breeding this species. As a consequence, aquaculture cannot fulfill the high demand for this fish. The farmers claimed a decrease of both quantity and quality of their production, and attributed it to loss of genetic variability and possible inbreeding. From the results reported here, it is now clear that this decline in production is not due to genetic factors and is probably caused by other problems such as poor water quality. It is now crucial to increase the aquaculture production of this species in order to quickly alleviate the overfishing pressure. This, of course, involves a better management of the quality of waters used for rearing *M. boesemani*, which is a general concern in Indonesia.

Finally, priority strategies for conservation of genetic diversity need to consider not only the level of diversity in an area but also the current threat to the condition of a particular region. Results obtained in the present study suggest that *in situ* conservation of the *Melanotaenia* complex should focus on metapopulations which have a higher level of genetic variation than populations from other groups within this species complex. For *ex situ* conservation, sampling more populations rather than intense sampling of a few populations is a preferable strategy. *Ex situ* conservation can be achieved through genetic management like captive breeding programs. It also has the advantage to provide people with economical value and high quality nutrients. All the species characterized in this manuscript deserve to

be immediately domesticated and cultivated in order to preserve them. They also represent possible new candidates in aquaculture commodity that may have high economy on the international market. Study of the population, distribution and genetic variation of *Melanotaenia* species should provide useful information in the active domestication of these species and allow prioritizing areas for future collection and conservation. Genetic diversity data are scarce for West Papua *Melanotaenia* species, and the genetic markers developed here should prove valuable for both *in situ* and *ex situ* conservation strategies.

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ANNEXE

ANNEXE 1

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TECHNICAL NOTES

Development of twelve novel polymorphic microsatellite DNA markers for the Boeseman's rainbowfish (*Melanotaenia boesemani*) and tests for their cross-utility in 21 rainbowfish species from West Papua (Indonesia)

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Abstract We developed 12 microsatellite markers for the endangered Boeseman's rainbowfish (*Melanotaenia boesemani*). Twenty-eight individuals from the type locality at Ayamaru Lake were examined, and all loci were polymorphic with a number of alleles per locus varying from 3 to 18. Average observed and expected heterozygosities were 0.681 and 0.678, respectively. Cross-species amplification was successfully obtained for 21 *Melanotaenia* species, with a number of alleles per locus ranging between 1 and 20. Average observed and expected heterozygosities varied between 0.105 and 0.708 and 0.118–0.755, respectively. Only 21 inbreeding coefficient (Fis) values presented a significant homozygote excess among the 264 locus-by-locus calculated values. Tests for genotyping errors revealed that four of these 21 significant Fis values could be explained by the presence of null alleles. These new microsatellite markers appear highly reliable for further conservation purposes or population genetic studies of the many rainbowfish endangered species.

Keywords Rainbowfish · Microsatellite DNA · *Melanotaenia boesemani* · West Papua · Endangered species

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Introduction

Rainbowfishes (Melanotaeniidae) are widely distributed throughout New Guinea, Australia, and the neigbouring islands. They are very popular for ornamental trade because of their vivid coloration and their ability to thrive in captivity (Kadarusman et al. 2010). They display amazing species richness, and some of them are on the red list of endangered species since 2004 (IUCN 2013), including *Melanotaenia ajamaruensis* Allen and cross 1980, *Melanotaenia angfa* Allen 1990, *Melanotaenia arfakensis* Allen 1995, 2007, 2008, *Melanotaenia boesemani* Allen 1995, 2007, 2008, *Melanotaenia catherinae* (de Beaufort, 1910), *Melanotaenia misoolensis* Allen, 1982 and *Melanotaenia parva* Allen, 1990. For illustration, overfishing of *M. boesemani* specimens conducted this species to the verge of extinction (Allen 2007; Kadarusman et al. 2012b). The first phylogenetic relationships of the Melanotaeniidae were inferred by sequencing a fragment of the mitochondrial cytochrome b gene (McGuigan et al. 2000). More recently, the use of other markers resulted in the description of many new species (Kadarusman et al. 2010, 2011, 2012a) and showed that the diversity of rainbowfishes in Western New Guinea (West Papua,

Indonesia) was largely underestimated (Kadarusman et al. 2012b). To date, the family consists of 84 species spread in seven genera, which are broadly distributed throughout the region. Western New Guinea is a center for rainbowfish diversity belonging to the *Melanotaenia* genus (Allen 1995; Allen et al. 2008; Kadarusman et al. 2010; McGuigan et al. 2000). Currently, 19 species have been described from this region: *M. ajamaruensis*, *M. ammeri* Allen et al. 2008, *M. angfa*, *M. arfakensis*, *M. arguni* Kadarusman et al. 2012a, b*M. batanta* Allen & Renyaan, 1996, *M. boesemani*, *M. catherinae*, *M. fasinensis* Kadarusman et al. 2010, *M. fredericki* (Fowler, 1939), *M. irianjaya* Allen, 1985, *M. kokasensis* Allen et al. 2008, *M. misoolensis*, *M. parva*, *M. salawati* Kadarusman et al. 2011, *M. synergos* Allen & Unmack, 2008, *M. urisa* Kadarusman et al. 2012a, b*M. veoliae* Kadarusman et al. 2012a, b, and *M. wanoma* Kadarusman et al. 2012a, b.

Considering their increasing economic value and the ever growing human pressures on their habitats, more research on the genetic variability of these fishes is needed to identify candidate species for aquaculture and for conservation purposes. To address this question, new microsatellite markers were developed and validated on *Melanotaenia* species present in Western New Guinea.

Methods

The investigated species included all *Melanotaenia* nominal species from Western New Guinea (with the exception of *M. kokasensis* for which no sample was available), one species from Central Lengguru (i.e., *Melanotaenia mairasi* Allen & Hadiaty, 2011) and three species from Southern New Guinea (i.e., *Melanotaenia goldiei* Macleay, 1883; *M. kamaka* Allen & Renyaan, 1996; *M. pierucciae* Allen & Renyaan, 1996). For most species, between 14 and 45 individuals were captured at a single location by using gillnets. Due to low population density, three species (i.e., *M. ammeri*, *M. fredericki*, *M. irianjaya*) were sampled at various locations in the same river drainage, with geographic distances varying from 1 to 5 km.

Tissue samples consisted of 1 cm^2 caudal fin preserved in absolute ethanol and stored at −20 °C. DNA was extracted using the NucleoSpin® 96 Tissue kit (Macherey-Nagel), according to the manufacturer's instructions, using a Janus automated Workstation (Perkin Elmer). It was eluted in 70 μl of the provided elution buffer, and its concentration was measured using a Nanodrop ND8000 spectrophotometer (Thermo Scientific). An equimolar mix of DNA extracted from seven individuals of *M. boesemani* was enriched for microsatellite motives and sequenced on a 454 GS-FLX Titanium sequencer (Roche) at Genoscreen (Lille, France), according to the previously described method (Malausa et al. 2011). Raw sequences were analyzed with the QDD program (Meglecz et al. 2010) for identification of microsatellite loci and primer design.

Microsatellite amplifications were carried out in a GeneAmp 2400 PCR cycler (Applied Biosystems), using the Fast-Start PCR kit (Roche). Each reaction contained 5 μl of 2× Master mix, 0.1 μM of fluorescently labeled forward primer (Eurofins), 0.4 μM of reverse primer, and 0.5 μl of template DNA, in a final volume of 10 μl. Cycling conditions were as follow: 95 °C for 4 min; 30 cycles of 95 °C for 20 s, 56 °C for 20 s and 72 °C for 30 s; and 7 min at 72 °C. PCR products were diluted 1:50 in H_2O and 1 μl of four dilutions were mixed together (according to Table 1) with 13.8 μl of formamide and 0.2 μl of GeneScan™ 600LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis was run in a 24-capillary sequencer (ABI Prism® 3500 XL), and allele sizing was achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems). Allelic diversity (Na), average expected (He), and average observed (Ho) heterozygoties were calculated using the GENETIX 4.05 software (Belkhir et al. 1996). Locus-by-locus and multilocus Hardy–Weinberg equilibrium was tested for each species by estimating the significance of the inbreeding coefficient (Fis) from sets of 1000 random allelic permutations of the original dataset, as implemented by GENETIX 4.05 software. The *p* value of Fis was defined as the probability to obtain absolute values higher than or equal to the observed one under the null hypothesis. In order to assess the possible presence of genotyping errors, the presence of null alleles, stuttering or large allele dropout, was also tested for all the analyzed loci across all the species with the Micro-Checker v. 2.2.3 software (CI 95 %, bootstrap simulation ×1000) (Van Oosterhout et al. 2004).

Results

Sequencing resulted in 14,922 sequences harboring microsatellites, for which 1012 primer sets could be designed. Twelve loci were selected according to several criteria (motif length, number of repeats, amplicon size, distance of primers from the repeated motif, amplification yield), and their characteristics are described in Table 1. Their polymorphism was evaluated on 586 fish specimens belonging to 22 nominal species. Except for locus Penta-06 that could not be amplified in *M. goldiei* DNA, all loci could be successfully genotyped in all tested species.

All microsatellite loci revealed polymorphic on the endangered *M. boesemani* with a number of alleles per locus varying from 3 to 18 (Table 1) and with an average observed and expected heterozygosity of 0.681 and 0.678, respectively (Table 2). Cross-species amplification was successfully

Table 1 Characteristics of the microsatellite markers on *Melanotaenia boesemani* and on the 21 cross-amplified species

Locus name	Gene bank no.	Primer sequence 5′-3′	Repeat motif[a]	Fluorescent label	CE mix[b]	S^c	S^d	A^e	A^f
Mb_di1	KF856886	F: TGAGTCAAGGGATGTCCAAA R: GATGTCCCTCCATAGCCTGT	(CA)23	5′-FAM	1	89–139	081–139	14	26
Mb_di2	KF856887	F: TCCCACCATGCATTATTAACAC R: GAACTGCAGGCTCAACACAT	(GT)18	5′-HEX	1	113–145	111–227	14	43
Mb_di3	KF856888	F: TGGAGGATTGTAGGTCTGGG R: CATCAACATAGCAATCAGTGCC	(GT)16	5′-ATO550	1	153–173	145–231	8	42
Mb_penta1	KF856889	F: TGTAACGAGTGAATTTCTCCACTG R: TCCACAATATTTACTGGAACTGC	(TCTAA)12	5′-ATO565	1	160–200	160–240	8	17
Mb_di4	KF856890	F: CAAGGCAGCAAAAGAACAAA R: CAGGCCATTCCTGAGTCAAT	(AC)20	5′-FAM	2	112–152	98–170	15	33
Mb_tetra1	KF856891	F: TTTACTACACGTGGTCTTCACTTT R: CGTTTTAGCCACAAACAGCC	(AATG)6	5′-ATO550	2	135–157	125–217	7	31
Mb_tri1	KF856892	F: CTGTCAGAGTGCAGGACTGG R: TCTTTTCCAGGCCAAGTGAC	(GGA)8	5′-ATO565	2	107–119	101–139	4	11
Mb_tri2	KF856893	F: CGTGTCTATTCCTATCGTGCC R: TGGATTCACATCCTCTTGAGTG	(GAG)11	5′-HEX	2	138–150	132–165	4	11
Mb_di5	KF856894	F: TCCTTGAATACATTCCTGCCA R: CAATAATTTCAAGGAAGAGCCTTT	(AC)16	5′-ATO565	3	085–099	073–127	8	28
Mb_tetra2	KF856895	F: GAAGAGTATGAAGAAGCCTTAGTCTGA R: CGTCCTGCAATCTACTGTGA	(CATC)18	5′-HEX	3	172–252	136–296	18	44
Mb_tri3	KF856896	F: TGACAGTAACAGGGACGATGA R: AAGGCAACCTAATGTGCTGT	(ATG)12	5′-ATO550	3	133–154	111–163	3	16
Mb_tri4	KF856897	F: AACAACAGGCAGGGAGTCAC R: CCAAGAGGTTGTGGTGTTCC	(ATG)10	5′-HEX	3	099–114	099–132	4	10

[a] According to the *M. boesemani* DNA used to construct the genomic DNA library

[b] Mix of amplification products used for capillary electrophoresis

[c] Allele size range (bp) for *M. boesemani*

[d] Allele size range (bp) for all 22 species

[e] Allele number for *M. boesemani*

[f] Allele number for all 22 species

obtained for 21 other *Melanotaenia* species with a number of alleles per locus ranging between 1 and 20 and average observed and expected heterozygosities varying from 0.105 to 0.708 and 0.118 to 0.755, respectively (Table 2). Considering all analyzed species, the total number of alleles per locus ranged from 10 to 44 (Table 1).

Only 21 Fis values presented significant homozygote excess among the 264 realized pairwise tests (Table 3). Multilocus Fis values revealed that three species (i.e., *M. fredericki*, *M. irianjaya*, *M. ammeri*) significantly departed from Hardy–Weinberg expectations (Table 3). The Microchecker analysis performed on the entire dataset (i.e. the 264 locus/species combinations) suggested the presence of null alleles for 2 loci (Mb_penta1 and Mb_di5) in 2 species, namely *M. fredericki* and *M. ammeri*. However, no evidence of scoring errors due to stuttering or large allele dropout was revealed across the entire dataset.

Discussion

The use of next generation sequencing enabled very quick development of microsatellite markers in species for which no or limited genomic resources were available. The 12 new markers described here proved efficient to genotype 22 species belonging to the *Melanotaenia* genus, among which seven species (*M. ajamaruensis*, *M. angfa*, *M. arfakensis*, *M. boesemani*, *M. catherinae*, *M. misoolensis*, and *M. parva*) are classified in the red list of IUCN. It is important to remind here that the only three species presenting significant multilocus Hardy–Weinberg deviations consisted of a mix of individuals sampled at various locations along the same river drainage. These results suggest the possible occurrence of genetically differentiated subpopulations patchily distributed in the same river and must be taken into consideration in future sampling strategies or population monitoring of

Table 2 Sample size (*N*), allele diversity (per species and per locus), and average observed (Ho) and expected (He) heterozygosity per species across loci

	N[a]	Mb_di1	Mb_di2	Mb_di3	Mb_penta1	Mb_di4	Mb_tetra1	Mb_tri1	Mb_tri2	Mb_di5	Mb_tetra2	Mb_tri3	Mb_tri4	Ho	He
M. ajamaruensis	30	9	12	8	7	10	6	3	5	5	11	3	3	0.670	0.636
M. fasinensis	40	4	5	9	3	4	6	1	1	2	8	1	1	0.365	0.351
M. arfakensis	45	6	3	12	9	1	5	2	1	2	11	6	1	0.404	0.398
M. boesemani	28	14	14	8	8	15	7	4	4	8	18	3	4	0.681	0.678
M. catherinae	37	2	1	1	3	1	2	1	1	1	2	1	3	0.146	0.144
M. synergos	39	2	1	1	3	1	3	1	1	2	3	2	1	0.191	0.186
M. parva	40	5	5	8	6	1	5	2	2	3	4	2	1	0.386	0.380
M. angfa	35	1	2	2	2	1	3	1	1	3	2	1	1	0.245	0.234
M. fredericki	11	7	19	17	10	3	12	3	6	12	9	4	4	0.657	0.710
M. irianjaya	23	13	14	17	9	6	14	8	5	10	12	7	5	0.708	0.755
M. veoliae	24	1	1	1	2	1	1	3	1	3	1	1	1	0.127	0.118
M. urisa	19	1	3	2	5	2	5	1	3	1	4	2	4	0.376	0.397
M. wanoma	14	1	2	4	4	3	5	3	3	2	3	1	2	0.387	0.369
M. ammeri	20	2	2	2	3	1	1	1	1	2	1	1	1	0.105	0.156
M. arguni	21	8	7	11	7	5	10	5	4	5	10	5	3	0.677	0.694
M. misoolensis	5	4	5	3	5	4	5	2	1	4	4	2	1	0.561	0.513
M. salawati	22	1	5	6	5	8	4	2	2	3	6	2	1	0.315	0.291
M. batanta	21	1	8	1	1	1	2	1	1	1	9	1	1	0.158	0.140
M. pierucciae	23	4	1	4	6	1	6	1	5	1	8	1	2	0.362	0.354
M. kamaka	25	7	1	9	9	2	9	1	7	2	20	2	2	0.469	0.458
M. goldiei	26	1	1	3	0	5	7	1	1	1	10	1	1	0.245	0.234
M. mairasi	30	4	6	4	9	7	10	1	1	15	3	1	1	0.372	0.377

[a] Number of individuals

Table 3 Locus-by-locus and multilocus Fis values for each species

	Mb_di1	Mb_di2	Mb_di3	Mb_penta1	M_di4	M_tetra1	M_tri1	M_tri2	Mb_di5	M_tetra2	Mb_tri3	M_tri4	Multi-locus
M. ajamaruensis	0.062	−0.046	−0.084	0.042	−0.104	−0.134	0.112	−0.128	−0.014	−0.036	−0.051	−0.072	−0.037
M. fasinensis	−0.113	0.127	−0.106	0.117	−0.247	−0.023	-	-	0.483**	−0.016	-	-	−0.027
M. arfakensis	0.072	−0.026	−0.036	0.019	-	0.036	0.000	-	−0.159	−0.018	0.055	-	−0.004
M. boesemani	0.099	0.074	−0.048	−0.137	−0.054	0.060	−0.110	0.112	0.002	0.106*	−0.105	0.017	0.013
M. catherinae	0.094	-	-	−0.078	-	0.107	-	-	-	−0.108	-	−0.115	−0.006
M. synergos	−0.019	-	-	−0.146	-	−0.033	-	-	−0.056	0.160	0.000	-	−0.010
M. parva	−0.045	−0.143	−0.074	0.088	-	0.028	−0.054	−0.068	0.047	0.089	0.000	-	−0.005
M. angfa	-	0.043	0.023	−0.087	-	−0.066	-	-	−0.028	−0.058	-	-	−0.032
M. fredericki	0.073	−0.023	−0.027	0.317**	0.024	0.016	0.232*	0.235*	0.309***	0.069	0.191	0.020	0.118***
M. irianjaya	0.140*	−0.013	−0.115	0.132*	0.173	0.015	−0.045	−0.032	0.159*	−0.077	0.685***	0.133	0.084**
M. veoliae	-	-	-	−0.007	-	-	−0.206	-	0.051	-	-	-	−0.048
M. urisa	-	0.030	−0.261	0.131	0.309*	0.090	-	0.034	-	−0.080	0.370*	0.186	0.080
M. wanoma	-	0.447**	0.274	−0.093	−0.056	−0.191	0.145	0.077	−0.083	−0.273	-	−0.040	−0.012
M. ammeri	0.088	−0.027	0.363***	0.347*	-	-	-	-	0.697***	-	-	-	0.351***
M. arguni	0.043	0.059	−0.033	0.191*	0.157*	0.013	0.077	−0.080	−0.032	0.054	0.129	−0.086	0.048
M. misoolensis	0.172	0.059	0.733***	0.059	0.070	0.143*	−0.429	-	−0.136	−0.351	−0.667	-	0.008
M. salawati	-	−0.012	0.005	−0.066	−0.118	−0.046	−0.050	−0.068	−0.028	−0.105	−0.024	-	−0.061
M. batanta	-	−0.087	-	-	-	−0.026	-	-	-	−0.132	-	-	−0.104
M. pierucciae	0.148	-	−0.166	0.078	-	−0.107	-	0.077	-	0.037	-	−0.143	−0.001
M. kamaka	−0.069	-	0.048	0.037	−0.070	−0.123	-	0.096	−0.086	−0.031	0.000	0.147	−0.003
M. goldiei	-	-	−0.009	-	0.062	0.009	-	-	-	−0.136	-	-	−0.026
M. mairasi	−0.043	0.080	−0.039	0.128*	0.081	−0.105	-	-	0.049	−0.040	-	-	0.030

- indicates a monomorphic locus

*$p<0.05$

**$p<0.01$

***$p<0.001$

Melanotaenia species. The majority of Hardy–Weinberg disequilibria reported for the different species were probably due to Wahlund effect rather than genotyping problems, as only four of them could be explained by null alleles.

Twelve microsatellite markers have already been described for the Australian species *Melanotaenia australis* (Castelnau, 1875) (Young et al. 2009). Tested on 32 individuals, these loci showed a moderate polymorphism, with a number of alleles ≤10 for all but one of them. Two of them showed a significant deviation from Hardy–Weinberg equilibrium, suggesting the possibility of null alleles.

To conclude, this new microsatellite resource is relevant for a wide number of rainbowfish species distributed on the New Guinea Island and will be extremely valuable not only for a better understanding of the diversification patterns of this fast-evolving group of fishes but also for the management of conservation strategies that are urgently needed.

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ANNEXE 2

Eight new species of Rainbowfishes (Melanotaeniidae) from the Birds Head Region, West Papua, Indonesia

by

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Abstract. – Eight new species of Rainbowfishes are described from the Birds Head region by combining a cytochrome oxydase I gene (COI) phylogeny, 12 microsatellite loci and diagnostic morphological characters. The new species *Melanotaenia klasioensis*, *M. longispina* and *M. susii* belong to the "Central Ayamaru Plateau" cluster in the COI phylogeny and are genetically allied to *M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi* and *M. fasinensis*. A Factorial Component Analysis made on the multilocus microsatellite genotypes reveals that these new species are genetically distinct from each other and from other species known from the area. With the same approach, we also describe *M. manibuii*, a species belonging to the "Northern Birds Head" mitochondrial clade and *M. sembrae*, a species genetically close to *M. multiradiata*. Three additional new species, *M. naramasae*, *M. rumberponensis* and *M. sikuensis*, belong to the "Birds Neck" cluster and are genetically allied to *M. angfa* and *M. parva*. The microsatellite markers were also used to distinguish *M. angfa* from *M. parva*, two species sharing the same mitochondrial haplotype. For the sake of comparison, the equivalent morphometric data are provided in appendix for the 20 valid species already described from the Birds Head area.

Résumé. – Huit nouvelles espèces de poissons arc-en-ciel (Melanotaeniidae) de la région de la Tête d'Oiseau en Papouasie occidentale, Indonésie.

Huit nouvelles espèces de poissons arc-en-ciel sont décrites de la région de la Tête d'Oiseau en combinant une phylogénie du gène cytochrome oxydase I (COI), 12 locus microsatellites et plusieurs caractères morphologiques diagnostiques. Les espèces nouvelles *Melanotaenia klasioensis*, *M. longispina* et *M. susii* appartiennent au cluster "Central Ayamaru Plateau" dans la phylogénie et sont génétiquement proches des espèces *M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi* et *M. fasinensis*. Une analyse factorielle des correspondances effectuée sur les génotypes microsatellites multilocus montre que ces espèces sont génétiquement différentiées les unes des autres ainsi qu'avec toutes les espèces présentes dans la région. Avec la même approche, nous décrivons également *M. manibuii*, une espèce appartenant au groupe phylogénétique "Northern Birds Head", ainsi que *M. sembrae*, une espèce génétiquement proche de *M. multiradiata*. Les 3 dernières espèces nouvelles que nous décrivons, à savoir *M. naramasae*, *M. rumberponensis* et *M. sikuensis*, appartiennent au cluster "Birds Neck" et sont phylogénétiquement apparentées à *M. angfa* et *M. parva*. Les marqueurs microsatellites ont également permis de différencier génétiquement *M. angfa* et *M. parva*, deux espèces qui possédaient le même haplotype mitochondrial.

Key words

Melanotaeniidae
Melanotaenia longispina
Melanotaenia susii
Melanotaenia klasioensis
Melanotaenia sembrae
Melanotaenia manibuii
Melanotaenia naramasae
Melanotaenia sikuensis
Melanotaenia rumberponensis
Australia
New Guinea
New species

Rainbowfishes of the family Melanotaeniidae are one of the most speciose group of freshwater fishes from the Australia-New Guinea region (Eschmeyer, 2014). They typically possess a compressed body covered by relatively large scales, two separate dorsal fins (the first with 3-7 spines and the second with a single spine and 6-22 segmented rays), a long-based anal fin, and no lateral line (Allen *et al.*, 2008). The family is characterized by relatively small (usually less than 10 cm) and often brightly coloured fish. Rainbowfishes are popular ornamental fishes owing to this coloration and their peaceful behaviour and ease of breeding. Sexual dimorphism is often apparent with males generally deeper-bodied

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and more vividly coloured than females (Allen, 1991). They are locally abundant and occupy a large array of freshwater habitats including arid-zone waterholes, swamps, rainforest streams and lakes. They are opportunist omnivores and show little ecological or morphological specialization like many other freshwater species of the Australian region (Roberts, 1978; McDowall, 1981).

Melanotaeniidae occur in tropical and subtropical Australia and one species occupies the southern Murray-Darling system. The family is also found throughout the island of New Guinea below about 1600 m elevation, as well as on most surrounding large islands of the western half of the mainland, including the Raja Ampat and Aru Archipelagos and Yapen Island (Allen, 1991).

According to Eschmeyer (2014) and Allen *et al.* (2014a, b), seven genera and 86 species have been described so far within Melanotaeniidae: *Cairnsichthys* Allen, 1980 (1 species), *Chilatherina* Regan, 1914 (11 species), *Glossolepis* Weber, 1907 (9 species), *Iriatherina* Meinken, 1974 (1 species), *Melanotaenia* Gill, 1862 (62 species), *Pelangia* Allen, 1998 (1 species), and *Rhadinocentrus* Regan, 1914 (1 species). The greater diversity of the family occurs on the island of New Guinea with 71 endemic species, while 13 species are restricted to Australia, and only two species are shared between both biogeographic entities (Allen and Hadiaty, 2013; Unmack *et al.*, 2013).

The tectonic history of the New Guinea region shows that the Birds Head Peninsula is a section of the Australian craton that became separated from the main continental mass during the Early Cretaceous (Pigram and Davies, 1987). At the same period, the main section of New Guinea was expanded by the collision of the northward-moving Indo-Australian plate with the westward-moving Pacific tectonic plate, and a series of smaller plates (Hamilton, 1988; Charlton, 2000; Hill and Hall, 2003). During the Late Miocene (*c.* 10-12 MYA), a northeast-southwest compression coupled with several subduction processes resulted in the uplift of the rugged Lengguru fold-and-thrust belt, which unified the Birds Head to the greater New Guinea (Bailly *et al.*, 2009). This geologic episode was concomitantly marked by the uplift of the current Central Highlands that extend east west through New Guinea (Hall, 2002). These intense tectonic and orogenic

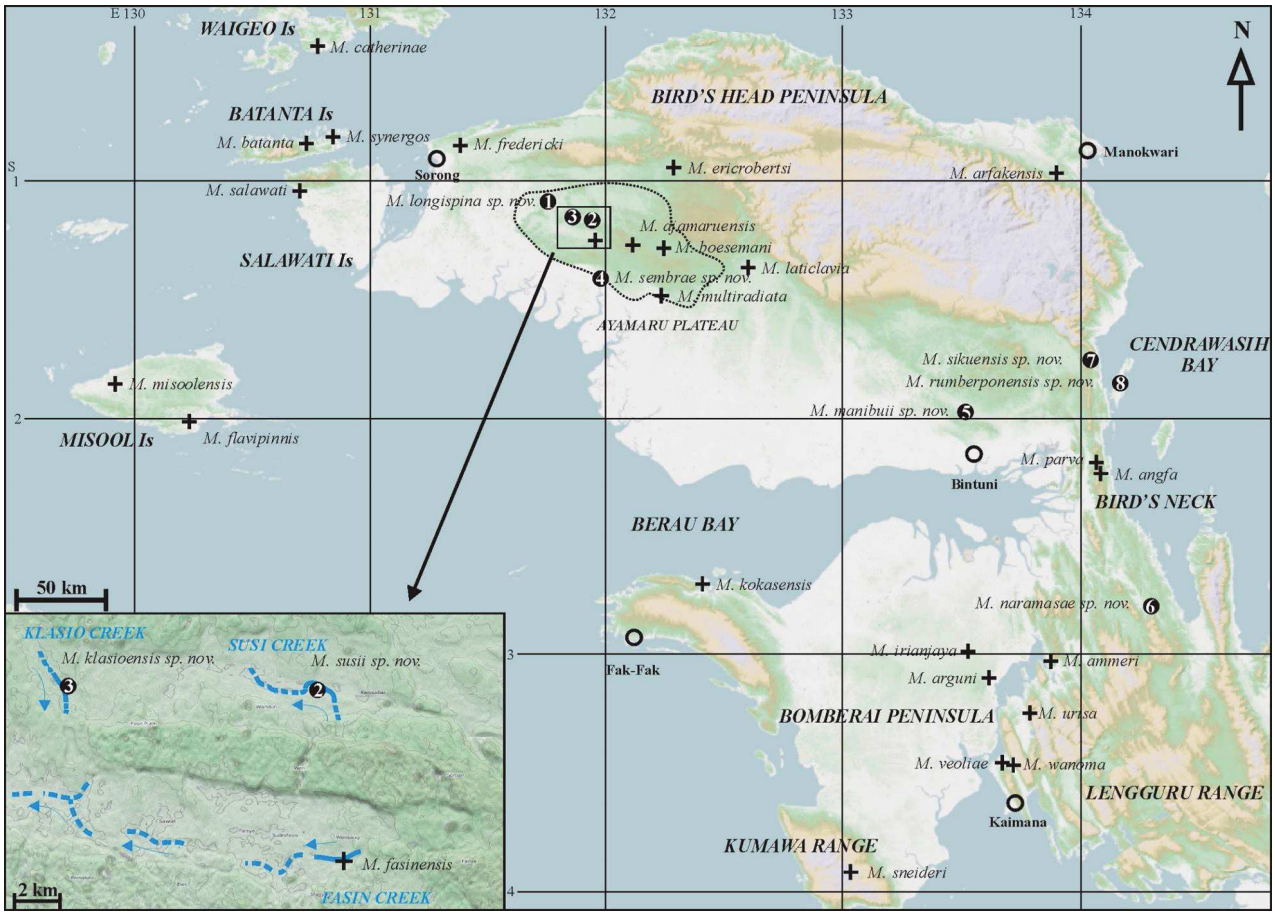

Figure 1. - Type localities of the 24 valid *Melanotaenia* species and the eight new species from the Birds Head region

Table I. - Rainbowfish species used in the barcode phylogenetic analysis and in the microsatellite marker genotyping; *, type locality; N_{COI}, sample size for barcode; GenBank # COI, accession number for barcode sequence; N_{SSR}, sample size for microsatellite markers.

Species	Locality	N_{COI}	GenBank # COI	N_{SSR}
M. ajamaruensis Allen & Cross, 1980	Kaliwensi R, Birds Head	3	KF491175-77	30
M. ammeri Allen *et al.*, 2008	Gusimawa*, Arguni Bay	3	KF491184-86	20
M. angfa Allen, 1990	Yakati R*, Birds Neck	3	KF491293, 16-17	36
M. arfakensis Allen, 1990	Prafi R*, Birds Head	3	KF491225, 27-28	45
M. arguni Kadarusman *et al.*, 2012	Jasu Ck*, Arguni Bay	3	KF491452-53, 56	22
M. batanta Allen & Renyaan, 1996	Warmon Ck*, Batanta Is	3	KF491239, 42-43	21
M. boesemani Allen & Cross, 1980	L Ayamaru*, Birds Head	3	KF491250-51, 55	28
M. catherinae (de Beaufort, 1919)	Waiwo Ck, Waigeo Is*	3	KF491258-60	37
M. ericrobertsi Allen *et al.*, 2014	Suswa*, Birds Head	2	KF491410-11	2
M. fasinensis Kadarusman *et al.*, 2010	Fasin Ck*, Birds Head	2	KF491265, 68	40
M. flavipinnis Allen *et al.*, 2014	Ifaupan Ck*, Misool Is		no sample	
M. fredericki (Fowler, 1939)	Sampson R*, Birds head	3	KF491284, 88-89	13
M. irianjaya Allen, 1985	Fruata*, Bomberai	3	KF491340, 46-47	24
M. kokasensis Allen *et al.*, 2008	Kokas Ck*, Bomberai		no sample	
M. klasioensis sp. nov.	Klasio Ck, Birds Head	3	KF491398-99, 03	40
M. laticlavia Allen *et al.*, 2014	Aifuf Ck*, Birds Head		no sample	
M. longispina sp. nov.	Klahfot R, Birds Head	2	KF491415-16	39
M. manibuii sp. nov.	Tisbo R, Birds Head	3	KF491440-41, 44	39
M. misoolensis Allen, 1982	Wai Tama*, Misool Is	3	KF491366-68	6
M. multiradiata Allen *et al.*, 2014	Moswaren*, Birds Head	3	KF491422, 26-27	40
M. naramasae sp. nov.	Naramasa R, Birds Neck	3	KF491213-15	29
M. parva Allen, 1990	L Kurumoi*, Birds Neck	3	KF491376-78	40
M. rumberponensis sp. nov.	Rumberpon Is. Birds Neck	3	KF491207, 09-10	17
M. salawati Kadarusman *et al.*, 2011	Doktor Ck*, Salawati Is	3	KF491390, 94-95	22
M. sembrae sp. nov.	Sembra R, Birds Head	3	KF491435-37	15
M. sikuensis sp. nov.	Siku Ck, Birds Neck	3	KF491203-05	42
M. sneideri Allen & Hadiaty, 2013	Small Ck in Kumawa*, Bomberai		no sample	
M. susii sp. nov.	Susi Ck, Birds Head	3	KF491417, 19, 21	31
M. synergos Allen & Unmack, 2008	Wai Bin Ck, Batanta Is*	3	KF491466-68	39
M. urisa Kadarusman *et al.*, 2012	L Sewiki*, Arguni Bay	3	KF491404-06	20
M. veoliae Kadarusman *et al.*, 2012	Gebiasi Ck*, Arguni Bay	3	KF491447-49	24
M. wanoma Kadarusman *et al.*, 2012	Wermura Ck*, Arguni Bay	3	KF491459-61	14

events resulted in the isolation of three major aquatic biotic provinces: northern New Guinea, the combined southern New Guinea/Australia, and the Birds Head region including the Lengguru Range (Allen, 1991; Abell *et al.*, 2008).

Kadarusman *et al.* (2012a) assessed the DNA barcoding diversity of 53 valid species of Melanotaeniidae throughout the Indo-Australian archipelago. This study confirmed the results of McGuigan *et al.* (2000) showing that *Melanotaenia*, along with *Chilatherina* and *Glossolepis* were subdivided into three main clades whose distribution is tightly matching the three biogeographic regions of New Guinea. Kadarusman *et al.* (2012a) added a fourth clade restricted to Lengguru Range.

In a recent study involving nuclear and mitochondrial DNA sequences, Unmack *et al.* (2013) confirmed that the three main clades correlated with the three main biogeographic regions of New Guinea, but failed to evidence the fourth clade restricted to Lengguru due to the lack of genetic samples (*i.e. Melanotaenia mairasi* Allen & Hadiaty, 2011).

Kadarusman *et al.* (2012a) therefore confirmed that the *Melanotaenia* species from the Birds Head region are monophyletic compared to the other main clades and highlighted the presence of nearly twice as many evolutionary lineages among the 15 valid species sampled in this area than previously described. These results revealed unexpected levels of cryptic diversity, demonstrating that the diversity of the family is still largely underestimated. Kadarusman and colleagues (2012a) concluded for Melanotaeniids that the biogeographic range of the Birds Head region should include the western side of Lengguru Range (*i.e.* all watersheds

flowing to Arguni Bay), the Bomberai and the Birds Head Peninsulas, the Birds Head Isthmus joining both peninsulas and the four major Raja Ampat islands (*i.e.* Misool, Batanta, Salawati, Waigeo).

The Birds Head region is a centre for rainbowfish diversity with 24 species currently described (Tab. I, Fig. 1).

A combination of molecular and morphologic approaches was used to determine differences between most of the currently recognised species and eight undetermined taxa that were recently obtained from the Birds Head region. We used partial sequences from the mitochondrial cytochrome oxydase I gene (COI) and 12 nuclear DNA microsatellites markers (Simple Sequence repeats – SSR) (Nugraha *et al.*, 2014) to assess whether morphological similar specimens from distinct locations are conspecifics or whether sufficient genetic variation and reproductive isolation exist to consider these specimens as distinct species.

The samples were collected during three expeditions conducted between 2007 and 2009 in West Papua by Balai Riset Budidaya Ikan Hias in Depok, Indonesia (BRBIH-KKP), the Akademi Perikanan Sorong, Indonesia (APSOR-KKP) and by the Institut de Recherche pour le Développement (Montpellier, France).

MATERIAL AND METHODS

DNA barcodes and tree-based species delimitation

Partial, 650-bp sequences from the 5' region of the COI were obtained from GenBank for 20 valid species and eight undetermined taxa according to Kadarusman *et al.* (2012a). Details about samples are given in figure 1 and table I. DNA samples were not available for *M. flavipinnis*, *M. kokasensis*, *M. laticlavia* and *M. sneideri*.

Phylogenetic analysis of the COI data set was inferred with neighbour joining (NJ) and maximum likelihood (ML) methods in MEGA5 (Tamura *et al.*, 2011). The Kimura 2-parameter (K2P) substitution model was used for the NJ analysis with 1000 bootstrap replicates to infer evolutionary distances. We also performed ML analyses with 500 bootstrap replicates using the Kimura 2-parameter model with a discrete Gamma distribution (K2P+G), which was the most appropriate nucleotide substitution model for our dataset based on the Bayesian Information Criterion (BIC) in the ML model selection feature of MEGA5.

Genotyping with nuclear DNA microsatellites

Individuals from the 20 valid species and the eight undetermined taxa described in table I were genotyped with 12 nuclear microsatellite markers that were recently developed (Nugraha *et al.*, 2014). Collected populations include the same localities and the same taxa analyzed at COI DNA barcodes (Tab. I; Fig. 1). Sampling strategy consisted in collecting each species at their type locality. For most of the species, between 13 and 48 individuals were captured per site, using landing nets. Because of low population density, five species (*i.e. M. ammeri*, *M. fredericki*, *M. irianjaya*, *M. manibuii* sp. nov., *M. naramasae* sp. nov.) were sampled at various localities in the same river drainage (geographic distances varying between 1 and 5 km) and collected individuals were pooled for subsequent analyses.

DNA was extracted from caudal fin clips preserved in absolute ethanol and stored at –20°C by using the NucleoSpin® 96 Tissue kit (Macherey-Nagel). DNA was extracted according to the manufacturer instructions using a Janus automated Workstation (Perkin Elmer). Microsatellite amplifications were performed with fluorescently labelled primers (Eurofins) and each reaction contained 5 µl of 2x Master mix (Roche), 0.1 µM of forward primer, 0.4 µM of reverse primer and 0.5 µl of template DNA. Cycling conditions were as follow: initial denaturation at 95°C for 4 min followed by 30 cycles of 95°C for 20 s, 56°C for 20 s and 72 °C for 30 s, and a final elongation step of 7 min at 72°C. Amplicon size was analyzed by capillary electrophoresis, in the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier). PCR products were diluted 1:50 in H_2O (or 1:100 in the case of fluorescence saturation). One µl of diluted PCR from 4 distinct fluorescent couples of primers were mixed together with 13.8 µl of formamide and 0.2 µl of GeneScan™ 600LIZ® Size Standard (Applied Biosystems). Capillary electrophoresis was run in an Applied ABI Prism® 3500 XL 24 capillary sequencer. Allele sizing and genotyping were achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems).

Allelic diversity and average observed (Ho) heterozygosity were calculated using GENETIX 4.05 (Belkhir *et al.*, 1996). Population gene pools were checked for departure from Hardy-Weinberg equilibrium by estimating the significance of the multilocus inbreeding coefficient (FIS) from sets of 1000 random allelic permutations of the original dataset as implemented by GENETIX 4.05 software. Significance levels of FIS *p*-values were tested using a one-tailed test as the probability of obtaining absolute values higher than or equal to the one observed under the null hypothesis (*i.e.* the individuals can be considered as the sample from a panmictic population at Hardy-Weinberg equilibrium). As the excess of homozygote can be partly explained by genotyping errors, the presence of null alleles, stuttering or large allele dropout was also tested for each species and locus-by-locus with the Micro-Checker, v. 2.2.3 software (Van Oosterhout *et al.*, 2004).

Genetic relationships between multilocus genotypes were assessed using factorial correspondence analysis (FCA) in GENETIX 4.05 software. Genetic structure among species was also investigated by calculating pairwise FST

values with 1000 random allelic permutations of the original dataset as implemented by GENETIX 4.05 software. Significance level of *p*-value for FST was defined as described above for FIS.

Species descriptions and specimen deposition

Specimens have been deposited at the Museum Zoologicum Bogoriense (MZB, Indonesia). The methods of meristic counts and measurements follow Allen and Cross (1980) with some modifications and additions (Kadarusman *et al.*, 2010). Measurements were performed with digital dial callipers under monocular lens (×2) and counts were made under binocular lens (×4). Measurements were made on the left side and are expressed to the nearest 0.1 mm. All proportions are expressed as percentage of the standard length.

Counts are as follow. – Lateral scales are the number of scales in horizontal row from the upper corner of the gill cover to the caudal fin base, excluding the small scales posterior to the hypural junction. Transverse scales are the number of scales in vertical row between the base of the first dorsal fin and the base of the anal fin origin. Predorsal scales are the number of scales along the midline of the nape in front of the first dorsal fin. Cheek scale is the total number of scales covering the suborbital and preoperculum. Dorsal rays are the number of spines in the first dorsal fin and the spine and soft rays in second dorsal fin. Anal rays are the single spine and number of soft rays. The last soft ray of the anal and second dorsal fins is divided at the base and counted as a single ray. Pectoral rays are the total number of segmented rays. Pelvic rays are the single spine and number of soft rays. Gillrakers are the total number on the first branchial arch.

Measurements are as follow. – Standard length is measured from the anteriormost tip of the upper lip to the posteriormost point of the hypural fold formed when the caudal peduncle is bent. Head length is measured from the tip of the upper lip to the upper rear edge of the gill opening. Snout length is the least distance measured from the tip of the upper lip to the fleshy anterior border of the eye. Interorbital width is the least width between the eyes anteriorly to the suture between frontal and nasal bones. Eye diameter is the maximal horizontal width of the orbital cavity. Body depth is measured from the base of the first dorsal spine to the base of the first anal spine. Body width is the maximal width measured posteriorly just behind the pectoral-fin base. Caudal peduncle depth is the minimum depth. Caudal peduncle length is measured from the base of the last dorsal fin ray to the vertebral-hypural junction at the caudal fin base. Predorsal length is measured from the tip of the upper lip to the base of the spine at the origin of first dorsal fin. Prepelvic length is measured from the tip of the upper lip to the base of the spine at the origin of pelvic fin. Preanal length is measured from the tip of the upper lip to the base of the spine at the origin of anal fin. Pectoral fin length is measured from the anteriormost part of pectoral fin base to the tip of the longest soft ray. Pelvic fin length is measured from the anteriormost part of pelvic fin base to the tip of the longest soft ray. Spine length of the first dorsal fin is measured from the base to the tip of the first spine on the first dorsal fin. Spine length of the second dorsal fin is measured from the base to the tip of the spine on the second dorsal fin. Spine length of the anal fin is measured from the base to the tip of the single anal spine. Dorsal fin base length is measured from the posterior base of the first spine of first dorsal fin to the posterior base of last soft ray of second dorsal fin. Second dorsal fin base length is measured on the second dorsal fin from the posterior base of the first spine to the posterior base of last soft ray. Anal fin base length is measured from the posterior base of the spine to the posterior base of the last soft ray. Anal fin height is measured from the base to the tip of the second soft ray.

Comparative material included 20 species described from the Birds Head region. This material includes type specimens and additional specimens collected at the type localities during three surveys (2007-2008-2010). These additional specimens were deposited at MZB, RMNH and MNHN. Their geographic locations are shown in figure 1.

Melanotaenia ajamaruensis. – Indonesia, West Papua. RMNH 28068 (holotype), 77.9 mm SL, Ajamaru; RMNH 28069-71 (paratypes), 7 specimens (57.0-63.8 mm SL), collected with holotype by Boeseman, 4-6 Mar. 1955; MZB 17692-94, 16 spms (62.5-96.5 mm SL), MNHN 2009-1617, 6 spms (55.5-66.5 mm SL), Kaliwensi River at Soroang village, 1°15.073'S, 132°08.156'E, collected by Kadarusman, Krenak, Paradis & Pouyaud, 22-23 May 2007.

Melanotaenia ammeri. – Indonesia, West Papua. MZB 16455 (holotype), 82 mm SL; Gusimawa village; MZB 16456 (paratypes), 4 spms (55.9-71.0 mm SL), collected with holotype by Allen & Ammer, 12 Jan. 2008; MZB 17709, 9 spms (62.4-101.8 mm SL), collected at type locality, 3°02.438'S, 133°52.844'E, Lemauk & Pouyaud, 4 Nov. 2010.

Melanotaenia angfa. – Indonesia, West Papua. MZB 17698, 3 spms (69.2-86.2 mm SL), MNHN 2009-1620, 5 spms (62.1-68.6 mm SL), RMNH.PISC.35675, 5 spms (59.5-62.6 mm SL), Pondok Creek, type locality, River Yakati, 2°11.067'S, 134°05.584'E, Kadarusman, Ajambua, Sumanta & Pouyaud, 11 Apr. 2008.

Melanotaenia arfakensis. – Indonesia, West Papua. MZB 17702, 3 spms (68.7-76.9 mm SL), MNHN 2009-1622, 4 spms (60.7-70.6 mm SL), RMNH.PISC.35677, 3 spms (60.9-71.3 mm SL), Supsan Creek, 0°58.376'S, 133°54.964'E, a tributary of Prafi River (type locality), Kadarusman, Ajambua, Paradis & Pouyaud, 30 May 2007.

Melanotaenia arguni. – Indonesia, West Papua. MZB 17712 (holotype), male, 54.9 mm SL, Egerwara village, Jasu Creek, 3°05.292'S, 133°37.879'E; MZB 17713 (paratypes), 6 spms (58.7-67.6 mm SL), MNHN 2010-0029 (paratypes), 4 spms (59.1-64.3 mm SL), RMNH.PISC.35876 (paratypes), 4 spms (58.9-

72.6 mm SL), collected with holotype by Kadarusman, Ruwe & Wamburie, 1 Nov. 2010.

Melanotaenia batanta. – Indonesia, West Papua. MZB 17705-06, 5 spms (88.2-99.2 mm SL), MNHN 2009-1625, 5 spms (79.5-107.9 mm SL), RMNH.PISC.35678, 5 spms (73.9-110.3 mm SL), type locality, Warmon stream, 0°50.256'S, 130°43.287'E, Bourhis, Sumanta & Pouyaud, 30 Apr. 2008.

Melanotaenia boesemani. – Indonesia, West Papua. RMNH 28061 (holotype), 67.2 mm SL, Ajamaru Lakes, Boeseman, 4 Mar. 1955; RMNH 28067 (paratypes), 2 spms (63.8-86.9 mm SL), same data as holotype; MZB 17691, 6 spms (54.7-60.7 mm SL) and MNHN 2009-1616, 4 spms (61.8-66.0 mm SL), Tiwit Creek, 1°15.463'S, 132°14.939'E, Kadarusman, Slembrouck & Pouyaud, 22 May 2007.

Melanotaenia catherinae. – Indonesia, West Papua. MZB 17703, 4 spms (65.4-79.8 mm SL) and MNHN 2009-1623, 4 spms (58.1-67.1 mm SL), Waiwo Creek on Waigeo Island (type locality), 0°25.060'S, 130°46.462'E, Kadarusman & Pouyaud, 14 May 2007.

Melanotaenia fasinensis. – Indonesia, West Papua. MZB 17700 (holotype), male, 108.5 mm SL, Ween village, Fasin Creek, 1°13.856'S, 131°58.186'E; MZB 17701 (paratypes), 4 spms (91.0-120.2 mm SL), MNHN 2009-1627 (paratypes), 4 spms (77.1-90.8 mm SL), RMNH.PISC.35680, 4 spms (69.9-78.5 mm SL), collected with holotype by Sumanta, Krenak, Kadarusman, Paradis & Pouyaud, 24 May 2007.

Melanotaenia fredericki. – Indonesia, West Papua. MZB 17695, 4 spms (65.0-71.9 mm SL), RMNH.PISC.35673, 4 spms (65.0-81.5 mm SL), MNHN 2009-1618, 4 spms (55.7-71.7 mm SL), Sampson River (or Warsamson), type locality, 0°49.361'S, 131°24.193'E, Kadarusman & Paradis, 13 Jun. 2007.

Melanotaenia irianjaya. – Indonesia, West Papua. MZB 4952 (holotype), 58.8 mm SL, Fruata; MZB 4953 (paratypes), 3 spms (39.6-57.8 mm SL), same data as holotype; MZB 17708, 10 spms (67.7-102.6 mm SL),

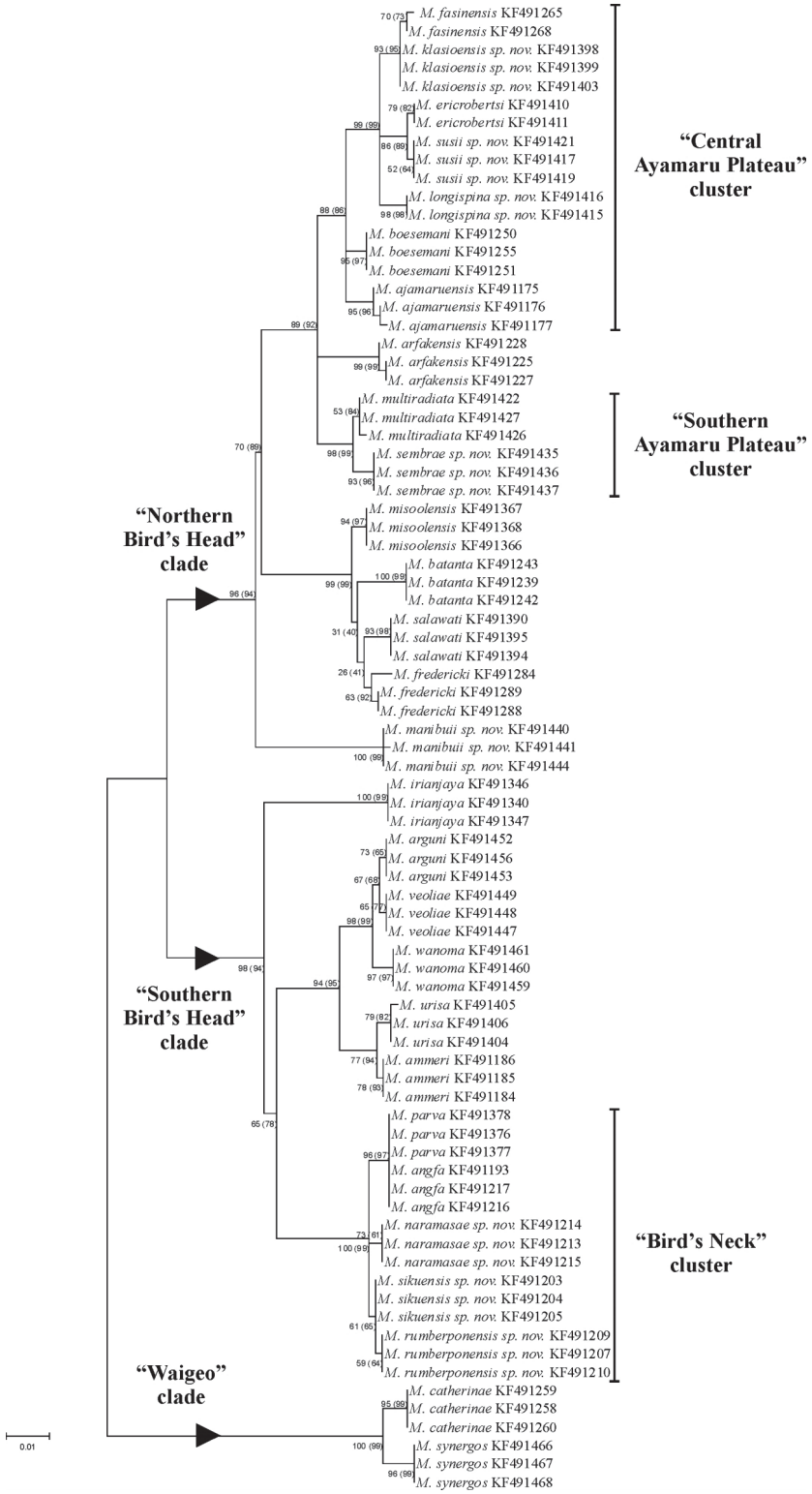

Figure 2. - Maximum likelihood tree for Birds Head region *Melanotaenia* species based on analysis of partial COI sequences (650 bp). Bootstrap values obtained for ML on 500 replicates and for NJ (within brackets).

Wat Creek, 2°58.576'S, 133°27.971'E, about 7 km from type locality, Kadarusman, 1 Nov. 2010.

Melanotaenia kokasensis. – Indonesia, West Papua. MZB 16453 (holotype), 57.2 mm SL, Kokas, Allen & Ammer, 16 Jan. 2008; MZB 16454 (paratypes), 2 spms (49.1-53.7 mm SL), same data as for holotype.

Melanotaenia misoolensis. – Indonesia, West Papua. MZB 17707, 3 spms (59.8-77.4 mm SL), RMNH.PISC.35679, 3 spms (46.1-48.9 mm SL), MNHN 2009-1626, 4 spms (54.0-58.4 mm SL), Wai Tama, type locality, 1°50.978'S, 129°54.654'E, Ajambua, Slembrouck, Sumanta & Pouyaud, 28 Apr. 2008.

Melanotaenia multiradiata. – Indonesia, West Papua. MZB 20033, 14 spms (64.1-89.9 mm SL), Waren R. at Moswaren, type locality, 1°29.158'S, 132°13.596'E, Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 23 Apr. 2008.

Melanotaenia parva. – Indonesia, West Papua. MZB 17699, 2 spms (65.4-67.0 mm SL), MNHN 2009-1621, 3 spms (57.7-62.0 mm SL), RMNH.PISC.35676, 3 spms (50.7-58.6 mm SL), Lake Kurumoi, 2°09.761'S, 134°05.155'E, collected at type locality by Kadarusman, Paradis, Sumanta, Pouyaud; 7-8 Jun. 2007.

Melanotaenia salawati. – Indonesia, West Papua. MZB 17710 (holotype), male, 88.7 mm SL, Waipule village, Doktor Creek, 1°01.128'S, 130°41.407'E; MZB 17711 (paratypes), 5 spms (71.4-91.6 mm SL), MNHN 2010-0030 (paratypes), 5 spms (68.0-83.6 mm SL), RMNH.PISC.35875, 5 spms (77.1-82.2 mm SL), collected with holotype by Sumanta, Ajambua, Slembrouck & Pouyaud, 29 Apr. 2008.

Melanotaenia synergos. – Indonesia, West Papua. MZB 17704, 2 spms (61.8-67.1 mm SL) and MNHN 2009-1624, 3 spms (57.7-63.5 mm SL), a small creek on north-eastern Batanta Island, less than 3 km from type locality, 0°50.288'S, 130°47.227'E, Kadarusman, Sumanta & Pouyaud, 15 May 2007.

Melanotaenia urisa. – Indonesia, West Papua. MZB 17715 (holotype), male, 71.4 mm SL, Sewiki Lake, 3°15.061'S, 133°47.735'E; MZB 20025 (paratypes), 6 spms (60.6-71.0 mm SL), MNHN 2010-0031 (paratypes), 5 spms (63.7-87.8 mm SL), RMNH.PISC.35877 (paratypes), 5 spms (57.4-62.0 mm SL), collected with holotype by Ajambua, Lemauk, Legendre & Pouyaud, 1 Nov. 2010.

Melanotaenia veoliae. – Indonesia, West Papua. MZB 20026 (holotype), male, 92.3 mm SL, Gebiasi Creek, 3°27.607'S, 133°41.071'E; MZB 20027 (paratypes), 5 spms (62.8-87.2 mm SL), MNHN 2010-0032, 5 spms (53.9-84.5 mm SL), RMNH.PISC.35878, 5 spms (64.5-65.0 mm SL), collected with holotype by Segura, Caruso, Setiawibawa, Sauri, Suruwaki, 7 Nov. 2010.

Melanotaenia wanoma. – Indonesia, West Papua. MZB 20028 (holotype), male, 71.9 mm SL, Wermura Creek, 3°28.261'S, 133°42.770'E; MZB 20029 (paratypes), 5 spms (62.8-72.8 mm SL), MNHN 2010-0033, 6 spms (59.6-65.0 mm SL), RMNH.PISC.35879, 6 spms (61.0-71.9 mm SL), collected with holotype by Segura, Caruso, Setiawibawa, Sauri, Suruwaki, 7 Nov. 2010.

Morphometric data corresponding to these species are given at the end of this paper (in appendix).

RESULTS

DNA barcodes and tree-based species delimitation

The ML tree obtained from COI mitochondrial sequences for 81 individuals belonging to 20 valid species and 8 new species (Tab. I) is presented in figure 2. The same topology was obtained for the K2P-NJ tree, thus, only bootstrap values (in brackets) were displayed together with those obtained for the ML tree.

Three major clades are highlighted by the ML tree (Fig. 2). These results are congruent with the topology of the ML tree based on cytochrome b provided in Allen *et al.* (2014b). The first clade "Northern Birds Head" (bootstrap: ML 96%, NJ 94%) includes all *Melanotaenia* species from the Birds Head Peninsula (*i.e. M. ajamaruensis*, *M. arfakensis*, *M. boesemani*, *M. ericrobertsi*, *M. fasinensis*, *M. fredericki*, *M. multiradiata*) and from the Southern Raja Ampat Islands (*i.e. M. batanta*, *M. misoolensis*, *M. salawati*). It also includes five new species (*i.e. M. klasioensis*, *M. susii*, *M. longispina*, *M. sembrae*, *M. manibuii*). The second clade "Southern Birds Head" (bootstrap: ML 98%, NJ 94%) includes all the *Melanotaenia* species from the Bomberai Peninsula (*i.e. M. ammeri*, *M. arguni*, *M. irianjaya*, *M. urisa*, *M. veoliae*, *M. wanoma*) and from the Birds Neck (*i.e. M. angfa*, *M. parva*). It also includes three new species (*i.e. M. naramasae*, *M. rumberponensis*, *M. sikuensis*). The third clade "Waigeo" is supported by high bootstrap values (ML 100%; NJ 99%) and includes the two species *M. catherinae* and *M. synergos* known from the Northern Raja Ampat (Fig. 1).

M. manibuii sp. nov. has a basal position in the first clade (Fig. 2) and displays an important phylogenetic distance with all the species composing this clade (average K2P distance: 0.053 ± 0.008). *M. manibuii* and *M. irianjaya* are morphologically closely related, although, they represent distinct species sorting in two different clades (*i.e.* first clade for *M. manibuii* and second clade for *M. irianjaya*) and are separated by a high phylogenetic distances (K2P distance: 0.069 ± 0.009).

The new species *M. sembrae* forms together with *M. multiradiata* the "Southern Ayamaru Plateau" cluster within the first clade (bootstrap: ML 98%, NJ 99%). The two species are separated by a low phylogenetic distance of less than 1% (K2P distance: 0.007 ± 0.003).

The new species *M. klasioensis*, *M. susii* and *M. longispina* belong to the cluster "Central Ayamaru Plateau" together with *M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi* and *M. fasinensis* (bootstrap: ML 88%, NJ 86%). These species are characterized by low phylogenetic distances (average K2P distance: 0.015 ± 0.003) and are all distributed in the fragmented river systems of the karstic Ayamaru Plateau. This cluster displays higher phylogenetic distances with *M. arfakensis* (average K2P distance: 0.030 ± 0.005) as well

as with the "Southern Ayamaru Plateau" cluster (average K2P distance: 0.029 ± 0.005).

Finally, the 3 new species *M. naramasae*, *M. sikuensis* and *M. rumberponensis* are genetically closely related with *M. angfa* and *M. parva*. They belong to the cluster "Birds Neck" and are characterised by phylogenetic distances of less than 1% (average K2P distance: 0.005 ± 0.002). This cluster, however, displays a higher phylogenetic distance with the remaining species composing the "Southern Birds Head" clade (K2P distance: 0.046 ± 0.007). Interestingly, the COI failed to differentiate *M. angfa* from *M. parva*, which display the same DNA barcode. This absence of genetic differentiation among these species was also revealed by Allen *et al.* (2014) for the cytochrome b gene and by Unmack *et al.* (2013) on one nuclear gene (S7) and seven mitochondrial markers (ND1, ND2, ND4L, ND4, ATPase6/8, cyt b, and partial COIII).

Nuclear DNA microsatellites

Twelve microsatellite loci were successfully amplified in 775 specimens including those analyzed by their COI DNA barcodes (Tab. I; Fig. 1). Characteristics of these loci, including allele size range and number per locus, are presented in table II. All microsatellite loci revealed polymorphism with a number of alleles per-locus ranging between 8 (locus Mb_tri2) to 53 (Mb_di2). The total number of allele per-species (Tab. III) indicates that *M. ericrobertsi* and *M. veoliae* are the species with the lowest level of polymorphism (13 and 17 alleles, respectively), while *M. multiradiata* and the new species *M. longispina* are the most polymorphic species (124 and 133 alleles, respectively). Average observed heterozygosity per species ranged between 0.042 and 0.743 (Tab. III). Most species were at panmictic equilibrium according to non-significant multilocus FIS values (Tab. III). Only five species (*i.e. M. ammeri*, *M. fredericki*, *M. irianjaya*, *M. manibuii*, *M. naramasae*) showed a significant deviation from Hardy-Weinberg equilibrium. No evidence for null alleles, scoring errors due to stuttering and for large allele dropout was revealed on the entire dataset according to Micro-Checker software. The five species presenting significant multilocus FIS values are a mix of individuals sampled at various locations along the same river drainage. These

Table II. - Characteristics of the 12 microsatellite markers used in the present study for 20 valid species and eight new species from the Birds Head region. S: Allele size range (bp) per locus for all species; A: Allele number per locus for all species.

Locus Name	Gene Bank #	Primer sequence 5'-3'	Repeat motif	S	A
Mb_di1	KF856886	F: TGAGTCAAGGGATGTCCAAA	CA	085-151	34
		R: GATGTCCCTCCATAGCCTGT			
Mb_di2	KF856887	F: TCCCACCATGCATTATTAACAC	GT	111-227	53
		R: GAACTGCAGGCTCAACACAT			
Mb_di3	KF856888	F: TGGAGGATTGTAGGTCTGGG	GT	148-231	48
		R: CATCAACATAGCAATCAGTGCC			
Mb_penta1	KF856889	F: TGTAACGAGTGAATTTCTCCACTG	TCTAA	150-240	18
		R: TCCACAATATTTACTGGAACTGC			
Mb_di4	KF856890	F: CAAGGCAGCAAAAGAACAAA	AC	098-172	38
		R: CAGGCCATTCCTGAGTCAAT			
Mb_tetra1	KF856891	F: TTTACTACACGTGGTCTTCACTTT	AATG	125-217	33
		R: CGTTTTAGCCACAAACAGCC			
Mb_tri1	KF856892	F: CTGTCAGAGTGCAGGACTGG	GGA	101-128	10
		R: TCTTTTCCAGGCCAAGTGAC			
Mb_tri2	KF856893	F: CGTGTCTATTCCTATCGTGCC	GAG	132-153	8
		R: TGGATTCACATCCTCTTGAGTG			
Mb_di5	KF856894	F: TCCTTGAATACATTCCTGCCA	AC	077-129	23
		R: CAATAATTTCAAGGAAGAGCCTTT			
Mb_tetra2	KF856895	F: GAAGAGTATGAAGAAGCCTTAGTCTGA	CATC	136-324	39
		R: CGTCCTGCAATCTACTGTGA			
Mb_tri3	KF856896	F: TGACAGTAACAGGGACGATGA	ATG	111-163	16
		R: AAGGCAACCTAATGTGCTGT			
Mb_tri4	KF856897	F: AACAACAGGCAGGGAGTCAC	ATG	098-132	13
		R: CCAAGAGGTTGTGGTGTTCC			

results therefore suggest the possible occurrence of genetically differentiated subpopulations patchily distributed in the same river.

Several factorial correspondence analyses (FCA) were conducted using microsatellite genotypes in order to assess the genetic differentiation between the new species and all other closely related valid species according to the COI phylogeny. The results are presented herein after:

(1) The three new species, *M. klasioensis*, *M. longispina* and *M. susii*, are genetically differentiated from each other as well as from *M. arfakensis* and all the remaining species that constitute the "Central Ayamaru Plateau" cluster (Fig. 3). All pairwise FST values show highly significant genetic divergence ($p < 0.001$) among these species (Tab. IV). Pairwise FST values between the three new species (*i.e. M. klasioensis*, *M. longispina*, *M. susii*) are comprised between 0.254 and 0.418. The new species *M. klasioensis* showed a low genetic distance with *M. fasinensis* on the COI (Fig. 2). The microsatellite data indicates that these two species constitute distinct genetic entities (Fig. 3) characterised by a significant FST value of 0.633 (Tab. IV). The same results were observed between *M. susii* and *M. ericrobertsi* and between *M. longispina* and all other species belonging to the "Central Ayamaru Plateau" cluster.

(2) The FCA presented in figure 4 shows that the new species *M. sembrae* is genetically differentiated from its sister species *M. multiradiata* (cluster "Southern Ayamaru Plateau"; Fig. 2). This new species is also genetically differ-

Table III. - Allele diversity (per species and per locus), average observed heterozygosity per species across loci, and per-species multilocus Fis values. Nall.: number of alleles per-species; Hobs.: average observed heterozygosity; Fis values significance: * $p < 0.01$; ** $p < 0.001$.

	Mb_di1	Mb_di2	Mb_di3	Mb_penta1	Mb_di4	Mb_tetra1	Mb_tri1	Mb_tri2	Mb_di5	Mb_tetra2	Mb_tri3	Mb_tri4	Nall.	Hobs	Fis multilocus
M. ajamaruensis	9	12	8	7	10	6	3	5	5	11	3	3	82	0.670	-0.037
M. ammeri	2	2	2	3	1	1	1	1	2	1	1	1	18	0.105	0.351**
M. angfa	1	2	2	2	1	3	1	1	3	2	1	1	20	0.245	-0.032
M. arfakensis	6	3	12	9	1	5	2	1	2	11	6	1	59	0.404	-0.004
M. arguni	8	7	11	7	5	10	5	4	5	10	5	3	80	0.677	0.048
M. batanta	1	8	1	1	1	2	1	1	1	9	1	1	28	0.158	-0.104
M. boesemani	14	14	8	8	15	7	4	4	8	18	3	4	105	0.681	0.013
M. catherinae	2	1	1	3	1	2	1	1	1	2	1	3	19	0.146	-0.006
M. ericrobertsi	1	1	1	2	1	1	1	1	1	1	1	1	13	0.042	–
M. fasinensis	4	5	9	3	4	6	1	1	2	8	1	1	45	0.364	-0.027
M. fredericki	7	19	17	10	3	12	3	6	12	9	4	4	106	0.657	0.118**
M. irianjaya	13	14	17	9	6	14	8	5	10	12	7	5	120	0.708	0.084*
M. klasioensis	1	15	6	2	3	4	2	1	2	3	1	1	41	0.310	-0.006
M. longispina	18	9	16	9	15	17	3	6	13	14	7	6	133	0.743	-0.003
M. manibuii	4	19	19	8	2	9	5	2	5	3	6	2	84	0.565	0.084**
M. misoolensis	4	5	3	5	4	5	2	1	4	4	2	1	40	0.561	0.009
M. multiradiata	13	10	23	9	19	13	3	3	4	18	7	2	124	0.608	0.020
M. naramasae	15	15	18	10	12	8	4	6	6	9	6	1	110	0.638	0.100**
M. parva	5	5	8	6	1	5	2	2	3	4	2	1	44	0.386	-0.005
M. rumberponensis	2	2	2	3	1	4	1	2	3	1	2	1	24	0.216	0.001
M. salawati	1	5	6	5	8	4	2	2	3	6	2	1	45	0.315	-0.061
M. sembrae	2	15	7	5	2	5	4	2	3	13	2	6	66	0.537	-0.015
M. sikuensis	2	3	2	2	1	3	2	2	1	1	2	1	22	0.149	0.068
M. susii	1	13	14	5	6	11	3	1	1	5	3	1	64	0.432	0.024
M. synergos	2	1	1	3	1	3	1	1	2	3	2	1	21	0.191	-0.010
M. urisa	1	3	2	5	2	5	1	3	1	4	2	4	33	0.376	0.080
M. veoliae	1	1	1	2	1	1	3	1	3	1	1	1	17	0.127	-0.048
M. wanoma	1	2	4	4	3	5	3	3	2	3	1	2	33	0.387	-0.012

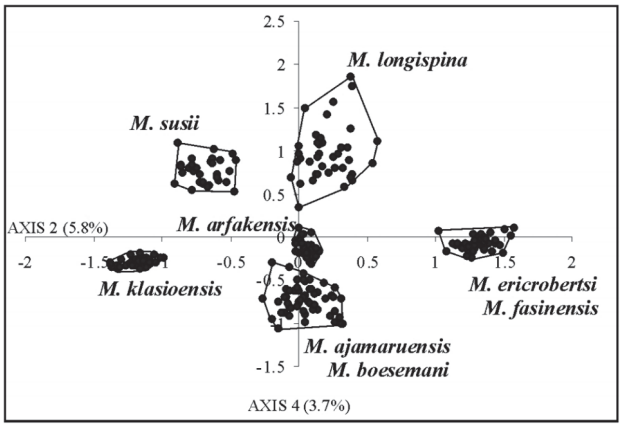

Figure 3. - Factorial correspondence analysis of genotypes (255 individuals; 12 loci) from all the species belonging to the phylogenetic cluster "Central Ayamaru Plateau" and to *M. arfakensis*. Projection on axis 2 and axis 4 segregating the new species *M. klasioensis*, *M. longispina* and *M. susii* with other species.

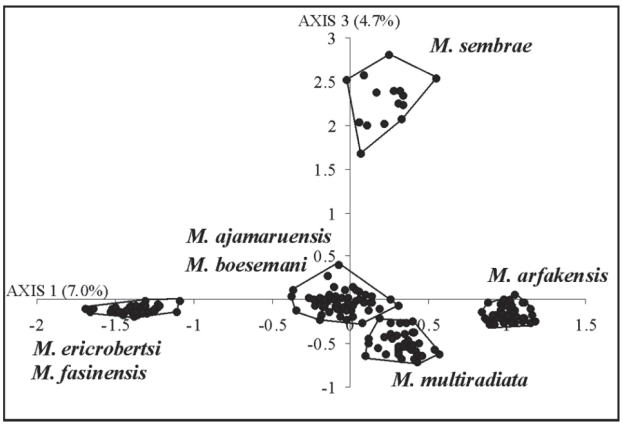

Figure 4. - Factorial correspondence analysis of genotypes (200 individuals; 12 loci) from all valid species belonging to cluster "Southern Ayamaru Plateau", to cluster "Central Ayamaru Plateau" and to *M. arfakensis*. Projection on axis 1 and axis 3 segregating *M. sembrae* sp. nov. with other species.

Table IV. - Pairwise FST values between species that form the cluster "Central Ayamaru Plateau" and *M. arfakensis*.

FST	*M. klasioensis*	*M. ajamaruensis*	*M. fasinensis*	*M. susii*	*M. arfakensis*	*M. boesemani*	*M. ericrobertsi*
M. longispina	0.407	0.191	0.354	0.254	0.374	0.171	0.338
M. klasioensis	–	0.398	0.633	0.418	0.597	0.361	0.707
M. ajamaruensis	–	–	0.415	0.348	0.427	0.072	0.418
M. fasinensis	–	–	–	0.587	0.595	0.412	0.231
M. susii	–	–	–	–	0.512	0.322	0.601
M. arfakensis	–	–	–	–	–	0.432	0.647
M. boesemani	–	–	–	–	–	–	0.393

Table V. - Pairwise FST values between species composing the cluster "Southern Ayamaru Plateau", all valid species from "Central Ayamaru Plateau" cluster, and *M. arfakensis*.

FST	*M. ajamaruensis*	*M. arfakensis*	*M. boesemani*	*M. sembrae*	*M. fasinensis*	*M. ericrobertsi*
M. multiradiata	0.238	0.415	0.198	0.355	0.498	0.488
M. ajamaruensis	–	0.427	0.072	0.288	0.415	0.418
M. arfakensis	–	–	0.432	0.484	0.596	0.647
M. boesemani	–	–	–	0.262	0.412	0.393
M. sembrae	–	–	–	–	0.517	0.524
M. fasinensis	–	–	–	–	–	0.231

entiated from *M. arfakensis* and from all other species (*i.e. M. ajamaruensis*, *M. boesemani*, *M. ericrobertsi*, *M. fasinensis*) belonging to the cluster "Central Ayamaru Plateau". All pairwise FST values computed between these species were highly significant ($p < 0.001$) and are given in table V.

(3) The FCA in figure 5 shows that the new species *M. manibuii* is genetically separated from *M. arfakensis*, *M. multiradiata*, *M. fredericki* and from all the species distributed in the Raja Ampat islands (*i.e. M. batanta*, *M. misoolensis*, *M. salawati*). In addition, the FCA presented in figure 6 indicates that *M. manibuii* is genetically separated from all the species composing the cluster "Central Ayamaru Plateau".

(4) The FCA obtained from the genotypes of the five new species (Fig. 7) confirms that *M. klasioensis*, *M. longispina*, *M. manibuii*, *M. sembrae* and *M. susii* are genetically differentiated from each other and therefore constitute reproductively isolated entities.

(5) The three new species *M. naramasae*, *M. rumberponensis* and *M. sikuensis* are genetically differentiated from each other, as well as from the remaining species *M. angfa* and *M. parva* that compose the "Birds Neck" cluster (Fig. 8). All pairwise FST values show highly significant genetic divergence ($p < 0.001$) among these species (Tab. VI). Pairwise FST values between the three new species are comprised between 0.356 and 0.714. Interestingly, *M. angfa* and

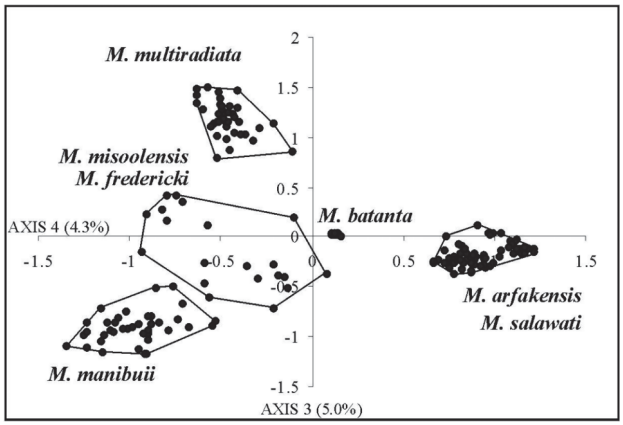

Figure 5. - Factorial correspondence analysis of genotypes (186 individuals; 12 loci) from *M. manibuii*, *M. arfakensis*, *M. multiradiata*, *M. fredericki*, *M. batanta*, *M. misoolensis*, *M. salawati*. Projection on axis 3 and axis 4 segregating *M. manibuii* sp. nov. with other species.

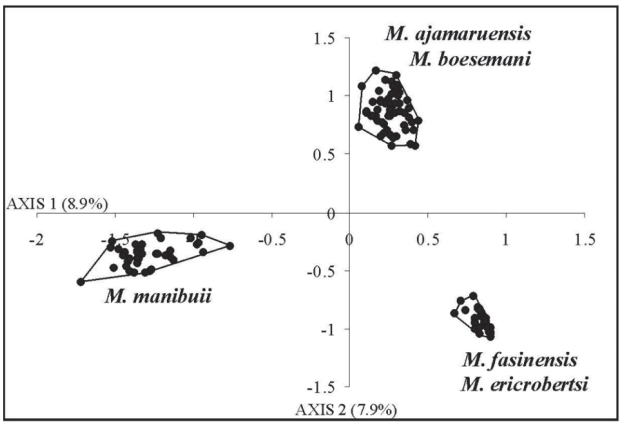

Figure 6. - Factorial correspondence analysis of genotypes (139 individuals; 12 loci) from *M. manibuii* and all valid species belonging to the cluster "Central Ayamaru Plateau". Projection on axis 1 and axis 2 segregating *M. manibuii* sp. nov. with other species.

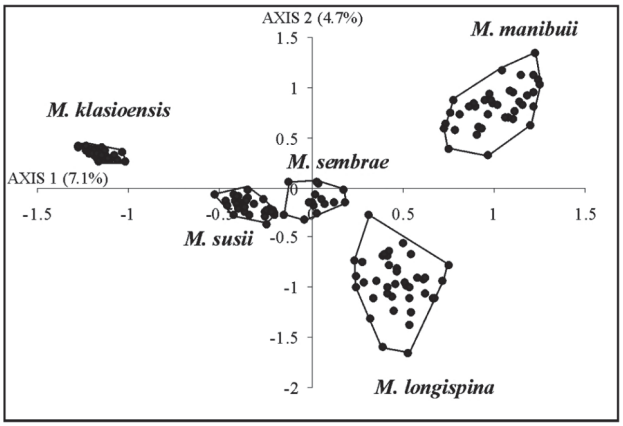

Figure 7. - Factorial correspondence analysis of genotypes (164 individuals; 12 loci) from the five new species belonging to COI "Northern Birds Head" clade. Projection on axis 1 and axis 2 segregating the five new species *M. klasioensis*, *M. longispina*, *M. manibuii*, *M. sembrae* and *M. susii*.

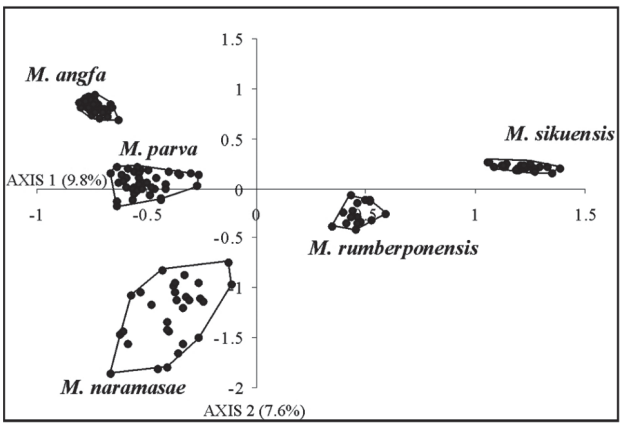

Figure 8. - Factorial correspondence analysis of genotypes (164 individuals; 12 loci) from all the species belonging to cluster "Birds Neck". Projection on axis 1 and axis 2 segregating the three new species *M. naramasae*, *M. rumberponensis* and *M. sikuensis* with other species composing this cluster.

Table VI. - Pairwise FST values between species composing the cluster "Birds Neck"

FST	*M. parva*	*M. angfa*	*M. rumberponensis*	*M. naramasae*
M. sikuensis	0.630	0.739	0.714	0.485
M. parva	–	0.460	0.545	0.245
M. angfa	–	–	0.707	0.401
M. rumberponensis	–	–	–	0.356

M. parva, the two species that share the same COI haplotype, displayed a significant pairwise FST value of 0.460. The locus Mb_tri1 revealed diagnostic for these two species, with a fixed allele (104) for *M. angfa* and two alleles [110 (75%) and 113 (25%)] for *M. parva*.

The microsatellite data therefore demonstrate that the eight new species represent distinct genetic entities, since they differ from each other and also from closely related valid species. Because no intermediate genotypes and no overlap were observed between these taxonomic entities, we therefore consider that these species represent new species whose morphological descriptions are given below.

SYSTEMATICS

Melanotaenia klasioensis, sp. nov.
Kadarusman, Hadiaty & Pouyaud
(Figs 9, 10; Tab. VII)

Type material

Holotype. – MZB 20034, male, 83.9 mm SL, 1°09.005'S, 131°51.927'E, a bridge on Klasio Creek near Malabolo village and on the road from Sorong to Ajamaru (Km 110), at the frontier post between Sorong and Sorong Selatan Regencies, Papua Barat Province, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 24 Apr. 2008.

Paratypes. – MZB 22151, 4 specimens (77.4-91.5 mm SL), same data as for holotype.

Diagnosis

Melanotaenia klasioensis is distinguished from all its congeners in the Birds Head region by the following combination of characters: dorsal rays V to VI + I,14 to 15; anal rays I,22 to 24; lateral scales 36 to 37; transverse scales 10; predorsal scales 17 to 18; cheek scales 16 to 17; total gillrakers on first arch 18 to 20; a large interorbital width, 8.6-9.2% of SL; a large body width, 14.2-15.8% of SL; a short preanal length, 47.7-49.4% of SL; a long pectoral fin length, 20.1-20.7% of SL; a long pelvic fin length, 19.4-22.6% of SL; 11.1% of SL; a prominent red margin stripe on dorsal fins; dorsal fin origin vertically projected behind anal fin origin, between 1st and 3rd anal soft ray.

Table VII. - Measurements taken on the holotype and four paratypes of *Melanotaenia klasioensis* sp. nov.

	Holotype	Paratypes				
SL (mm)	83.9	77.2-91.5				
in % standard length		n	Min	Max	Mean	SD
Head length	25.9	4	24.5	25.3	24.8	0.3
Snout length	9.1	4	8.6	9.1	8.9	0.2
Interorbital width	9.2	4	8.6	8.9	8.7	0.1
Eye diameter	6.9	4	6.5	7.4	6.9	0.4
Body depth	37.7	4	30.4	35.2	33.1	2.1
Body width	14.5	4	14.2	15.8	14.9	0.7
Caudal peduncle depth	11.4	4	10.2	10.7	10.5	0.2
Caudal peduncle length	14.9	4	14.7	15.8	15.2	0.5
Predorsal length	49.4	4	49.6	50.2	49.9	0.3
Prepelvic length	39.3	4	37.2	37.6	37.4	0.2
Preanal length	49.4	4	47.7	48.4	48.2	0.3
Pectoral fin length	20.2	4	20.1	20.7	20.3	0.3
Pelvic fin length	22.6	4	19.4	19.8	19.6	0.2
Spine length of first dorsal fin	10.4	4	10.4	10.9	10.7	0.2
Spine length of second dorsal fin	9.1	4	9.8	11.1	10.5	0.6
Spine length of anal fin	7.1	4	7.5	7.9	7.7	0.2
Dorsal fin base length	39.5	4	38.7	40.3	39.4	0.8
Second dorsal fin base length	26.2	4	24.5	26.1	25.3	0.8
Anal fin base length	45.0	4	39.1	40.7	39.9	0.7

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 4 specimens, 77.2-91.5 mm SL) if different from the holotype. Morphometric data are given in table VII.

Dorsal rays V + I,14 (V-VI + I,14-15); anal rays I,24 (I,22-24); pectoral rays 14 (13-15); pelvic rays I,5; lateral scales 37 (36-37); transverse scales 10; predorsal scales 17 (17-18); cheek scales 17 (16-17); total gillrakers on first arch 18 (18-20); jaws equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips; teeth at front of upper jaw in about 5-6 irregular rows, reduced to 1-2 rows posteriorly; teeth at front of lower jaws in about 7-8 irregular rows, reduced to 1-2 rows posteriorly; small conical teeth on vomer and palatines.

Scale of body large, cycloid, crenulated, arranged in regular horizontal rows; predorsal scales extending to posterior margin of interorbital; 3 rows of preopercle scales. First dorsal fin origin projected behind anal fin origin, between 1st and 3rd anal soft ray; tip of first dorsal fin reaching base of spine to 2nd soft ray of second dorsal fin; tip of second dorsal fin reaching half to ¾ of caudal peduncle in females and ¾ to hypural junction in males; tip of anal fin reaching 1/3 to half of caudal peduncle in females and half to ¾ of caudal peduncle in males; pelvic fin tips reaching base of 3rd to 6th anal soft ray in males and 2nd to 4th in females; caudal fin moderately forked.

Colour of freshly collected male specimens: overall body dark mauve; brown dorsally; lateral flanks mauve on upper half and yellowish on lower half; belly whitish; a continuous dark green midlateral stripe of 1 scale wide (2 scales on caudal peduncle) and sometimes interrupted on anterior part by 1-3 vertical bars (never exceeding 5 scales in length); pectoral fin translucent with black rays; pelvic fin yellow; anal fin overall dark with black rays separated by red hues; dorsal fins black with a marked white or red margin. Colour pattern of females is less vivid: overall body yellowish; a faint dark green midlateral stripe of 1 scale wide; no vertical bars; anal fin overall brown with green rays; dorsal fins grey with a narrow pink margin stripe.

Sexual dimorphism: Males are deeper bodied than females (35.2-37.7 *vs* 30.4-34.2% SL) and have second dorsal and anal fins more elongated posteriorly.

Comparisons

The new species *M. klasioensis* is part of the phylogenetic "Northern Birds Head" group and belongs

to the cluster "Central Ayamaru Plateau", together with the valid species *M. fasinensis*, *M. ericrobertsi*, *M. boesemani* and *M. ajamaruensis* (Fig. 2). *Melanotaenia klasioensis* is genetically close to *M. fasinensis* on the COI phylogeny but is easily distinguishable from the latter by the variability of analysed microsatellite loci (Figs 3, 7).

Melanotaenia klasioensis differs from *M. fasinensis* by more transverse scales (10 *vs* 9), fewer anal soft rays (22-24 *vs* 24-27), a greater interorbital width (8.6-9.2 *vs* 7.6-8.2% SL), a greater body width (14.2-15.8 *vs* 12.1-14.2% SL), a longer pectoral fin length (20.1-20.7 *vs* 17.4-19.9% SL) and fewer lateral scales (36-37 *vs* 37-39.

Following Allen *et al.* (2014b), *Melanotaenia klasioensis* can be distinguished from *M. ericrobertsi* by fewer lateral scales (36-37 *vs* 37-39), more cheek scales (16-17 *vs* 12-16), the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 3rd anal soft ray *vs* dorsal fin origin about equal with anal fin origin).

Melanotaenia klasioensis differs from *M. ajamaruensis* and *M. boesemani* by more transverse scales (10 *vs* 7-8), more predorsal scales (17-18 *vs* 15-16), a shorter preanal length (47.7-49.4 *vs* 49.4-57.6% SL), a longer pectoral fin length (20.1-20.7 *vs* 16.9-19.9% SL), a longer pelvic fin length (19.4-22.6 *vs* 13.8-18.2% SL).

Melanotaenia klasioensis differs from *M. laticlavia* by fewer soft anal rays (22-24 *vs* 24-26), more gillrakers (18-20 *vs* 16-17), the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 3rd anal soft ray *vs* dorsal fin origin about level with anal fin origin). *Melanotaenia laticlavia* belongs to the "Southern Birds Head" phylogenetic group following Allen *et al.* (2014b) and is genetically distant from the species belonging to the "Northern Birds Head" group. Nevertheless, this species is geographically close to the species inhabiting the Central Ayamaru Plateau, as it is distributed in the upper reaches of the Kamundan River system lying just on the eastern side of the Ayamaru Plateau (Fig. 1).

Distribution and habitat

The new species is currently known only from Klasio Creek, a karst resurgence of less than 5 m width with a course of a few kilometres before disappearing in a subterranean outlet probably connected to the underground course of the Fasin River (Fig. 1). The habitat in the vicinity of Malabolo village (Fig. 11) consists of a crystalline stream, relatively shallow (up to about 1 m), and flowing over white sand and limestone boulders in secondary forest.

Etymology

The species is named *klasioensis*, with reference to the creek name at the type locality.

Melanotaenia longispina, sp. nov.
Kadarusman, Avarre & Pouyaud

(Figs 12, 13; Tab. VIII)

Type material

Holotype. - MZB 22152, female, 78.7 mm SL, 1°04.160'S, 131°45.595'E, a bridge on Klahfot R., near Saluk village, Km 92 on road from Sorong to Teminabuan, Sorong Regency, Papua Barat Province, Indonesia. Sumanta, Krenak, Kadarusman, Paradis & Pouyaud, 24 Apr. 2008.

Paratypes. – MZB 22153, 3 specimens (61.3-73.3 mm SL), same data as for holotype; MZB 22154, 5 specimens (71.1-86.9 mm SL), collected at type locality by Sumanta, Kadarusman, Ajambua, Slembrouck & Pouyaud, 19 May 2007.

Diagnosis

Melanotaenia longispina is distinguished from all of its congeners in the Birds Head region by the following combination of characters: dorsal rays IV to VI + I,13 to 14; anal rays I,23 to 26; lateral scales 37 to 40; transverse scales 10; predorsal scales 19 to 21; cheek scales 17 to 18; total gill-

Table VIII. - Measurements taken on the holotype and eight paratypes of *Melanotaenia longispina* sp. nov.

	Holotype	Paratypes				
SL (mm)	78.7	61.3-86.9				
in % standard length		n	Min	Max	Mean	SD
Head length	24.7	8	24.1	25.2	24.5	0.4
Snout length	9.1	7	8.6	9.0	8.8	0.1
Interorbital width	7.9	8	7.8	8.2	8.0	0.2
Eye diameter	7.8	7	7.5	8.8	8.0	0.5
Body depth	30.4	8	26.2	33.9	31.2	2.7
Body width	11.5	8	11.7	13.2	12.2	0.6
Caudal peduncle depth	10.0	8	9.2	10.7	10.0	0.5
Caudal peduncle length	16.2	7	15.4	16.7	16.1	0.5
Predorsal length	51.7	8	50.2	51.8	51.1	0.6
Prepelvic length	36.7	8	35.5	36.7	36.2	0.5
Preanal length	48.3	8	46.0	48.2	47.3	0.8
Pectoral fin length	20.5	3	18.3	18.5	18.4	0.1
Pelvic fin length	19.9	3	19.6	20.4	20.1	0.4
Spine length of first dorsal fin	15.3	6	13.1	14.8	13.6	0.6
Spine length of second dorsal fin	15.7	7	12.4	15.5	13.7	1.4
Spine length of anal fin	11.3	5	10.1	10.4	10.3	0.1
Dorsal fin base length	33.4	8	34.1	36.5	35.5	1.0
Second dorsal fin base length	20.2	8	20.5	22.6	21.9	0.8
Anal fin base length	39.8	8	38.8	43.2	41.1	1.6

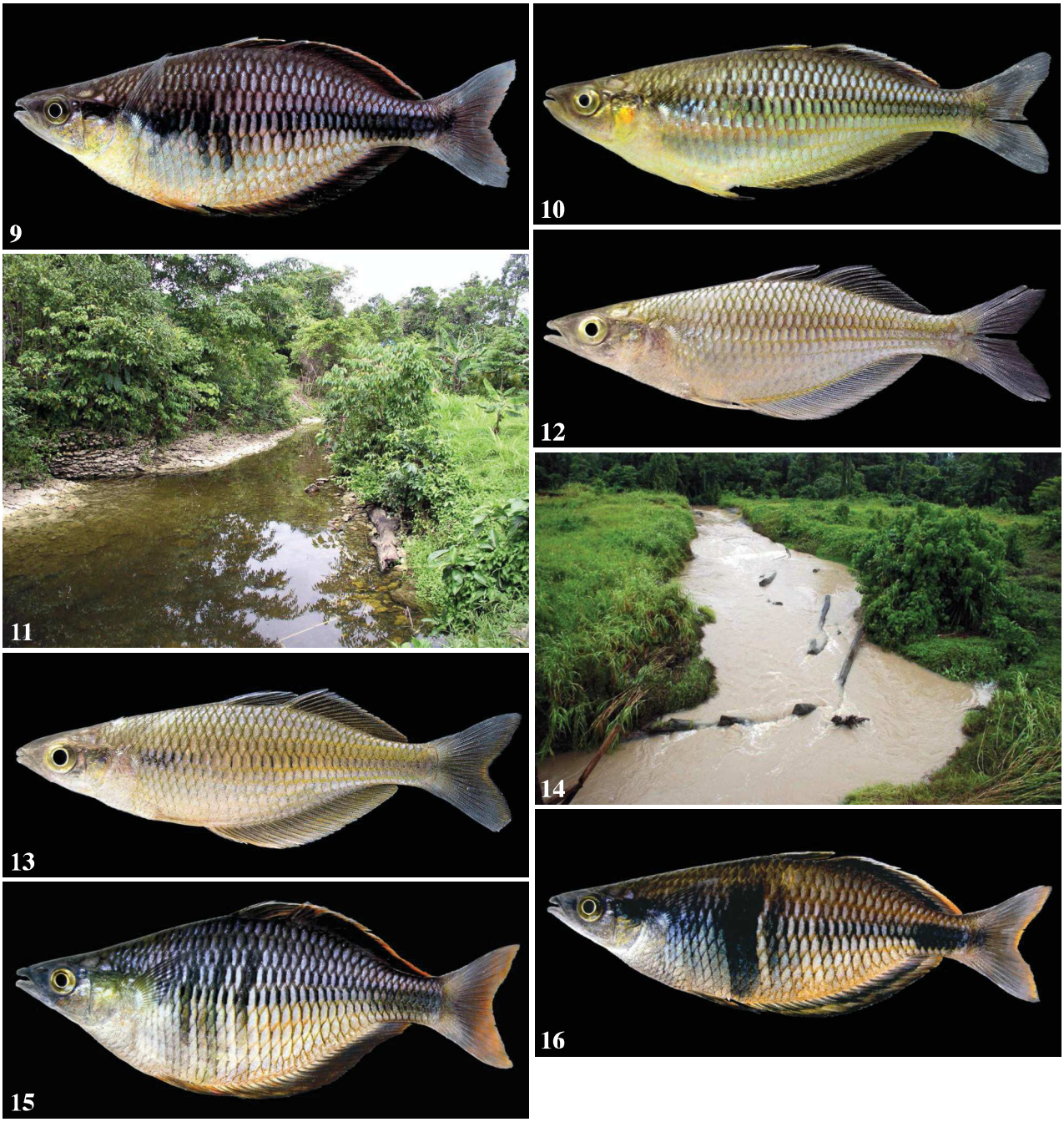

Figure 9. - *Melanotaenia klasioensis*, a male, 79 mm SL, Malabolo, Sorong Selatan, West Papua, Indonesia.
Figure 10. - *Melanotaenia klasioensis*, a female, 71 mm SL, Malabolo, Sorong Selatan, West Papua, Indonesia.
Figure 11. - Type locality of *Melanotaenia klasioensis*, Klasio Creek, near Malabolo, Sorong Selatan, West Papua, Indonesia.
Figure 12. - *Melanotaenia longispina*, MZB 22152 (holotype), female, 78.7 mm SL, near Saluk, Sorong Regency, West Papua, Indonesia.
Figure 13. - *Melanotaenia longispina*, MZB 22153 (paratype), male, 73.3 mm SL, same data as for holotype.
Figure 14. - Type locality of *Melanotaenia longispina*, Klahfot River, near Saluk, Sorong Regency, West Papua, Indonesia.
Figure 15. - *Melanotaenia susii*, MZB 22155 (holotype), male, 114.1 mm SL, Susi Creek, Sorong Selatan, West Papua, Indonesia.
Figure 16. - *Melanotaenia susii*, MZB 22156 (paratype), female, 111.7 mm SL, same data as for holotype.

rakers on first arch 19; a short head length, 24.1-25.2% of SL; a thin interorbital width, 7.8-8.2% of SL; a long predorsal length, 50.2-51.8% of SL; a long pelvic fin length, 19.6-20.4% of SL; a long spine length on first dorsal fin, 13.1-15.3% of SL; relatively elongate second dorsal fin spine, 12.4-15.7% of SL; elongate anal fin spine, 10.1-11.3% of SL; a short dorsal fin base, 33.4-36.5% of SL; a short second dorsal fin base, 20.2-22.6% of SL; no stripe on margin of dorsal fins; dorsal fin origin vertically projected behind anal origin, between 1st and 3rd anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 8 specimens, 61.3-86.9 mm SL) if different from the holotype. Morphometric data are given in table VIII.

Dorsal rays V + I,13 (IV-VI + I,13-14); anal rays I,23 (I,23-26); pectoral rays 15; pelvic rays I,5; lateral scales 38 (37-40); transverse scales 10; predorsal scales 20 (19-21); cheek scales 18 (17-18); total gillrakers on first arch 19; snout pointed and rounded dorsally; jaws equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips; teeth at front of upper jaw in about 4-5 irregular rows, reduced to 1-2 rows posteriorly; teeth at front of lower jaws in about 7-9 irregular rows, reduced to 1-2 rows posteriorly; small conical teeth on vomer and palatines.

Scale of body cycloid, large, with crenulated margin and arranged in regular horizontal rows; predorsal scales extending to posterior half of interorbital; preopercle scales arranged in 3 rows. First dorsal fin origin vertically projected behind anal fin origin, between the 1st and 3rd anal soft ray; tip of first spine of first dorsal fin reaching base of first spine of second dorsal fin; tip of second dorsal fin reaching half of caudal peduncle; pelvic fin tips reaching base of 1st to 5th anal soft ray; soft dorsal and anal fins triangular in outline; caudal fin moderately forked.

Colour of freshly collected male specimens: Overall body pale with pinkish and greenish reflections; series of 7-8 narrow and discrete yellowish stripes between each horizontal scale row; pale gray to bluish dorsally and whitish to pale pink ventrally; a midlateral stripe faint or absent in middle part of the body and sometimes only visible directly behind pectoral fin base and at the extremity of the caudal peduncle; pectoral fin translucent; remaining fins pink in males and whitish to translucent in females. No margin stripe on dorsal and anal fins. Overall colour of females is paler than males with darker narrower stripes between each horizontal scale row.

Sexual dimorphism: although some overlap is apparent, males are usually deeper bodied than females (28.7-33.9 *vs* 26.2-30.4% SL).

Comparisons

Melanotaenia longispina belongs to the "Central Ayamaru Plateau" group of species together with *M. fasinensis*, *M. ericrobertsi*, *M. boesemani* and *M. ajamaruensis* and the new species *M. klasioensis*. (Fig. 2). The new species differs from all of these species by the genetic polymorphism assessed from the 12 microsatellite loci (Figs 3, 7).

Melanotaenia longispina is easily distinguishable from all of these species by its lack of markings on the dorsal fin (present in other species).

In addition, the new species differs from *M. ajamaruensis*, *M. boesemani* and *M. fasinensis* by more transverse scales (10 *vs* 7-9), a longer pelvic fin length (19.6-20.4 *vs* 13.8-19.5% SL), a longer spine length on first dorsal fin (13.1-15.3 *vs* 8.6-12.7% SL), a longer spine length on second dorsal fin (12.4-15.7 *vs* 7-11.6% SL), a longer spine length on anal fin (10.1-11.3 *vs* 6.6-9.7% SL). The new species differs from *M. ajamaruensis* and *M. fasinensis* by a longer predorsal length (50.2-51.8 *vs* 45.3-50.0% SL), a shorter dorsal fin base length (33.4-36.5 *vs* 38.6-43.5% SL), and a shorter second dorsal fin base length (20.2-22.6 *vs* 24.1-29.5% SL). The new species differs from *M. boesemani* by a shorter head length (24.1-25.2 *vs* 25.5-28.5% SL), a narrow interorbital width (7.8-8.2 *vs* 8.3-9.5% SL), and a slender body depth (26.2-33.9 *vs* 34.0-44.7% SL).

Following Allen *et al.* (2014b), *Melanotaenia longispina* is distinguishable from *M. ericrobertsi* and *M. laticlavia* by more predorsal scales (19-21 *vs* 16-19), by the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 3rd anal soft ray *vs* dorsal fin origin about equal with anal fin origin). The new species differs from *M. laticlavia* by fewer soft dorsal rays (13-14 *vs* 14-17), more lateral scales (37-40 *vs* 36-37) and more gillrakers (19 *vs* 16-17). It differs from *M. ericrobertsi* by more cheek scales (17-18 *vs* 12-16).

Melanotaenia longispina differs from *M. klasioensis* sp. nov. by a greater eye diameter (7.5-8.8 *vs* 6.5-7.4% SL), a shorter dorsal fin base length (33.4-36.5 *vs* 38.7-40.3% SL), a shorter second dorsal fin base length (20.2-22.6 *vs* 24.5-26.2% SL), and by longer spines respectively on first dorsal fin (13.1-15.3 *vs* 10.4-10.9% SL), on second dorsal fin (12.4-15.7 *vs* 9.1-11.1% SL), on anal fin (10.1-11.3 *vs* 7.1-7.9% SL).

Distribution and habitat

The new species is currently known from the Klahfot River. This river ranged from about 10 to 15 m in width at the type locality (Fig. 14) and was flowing through secondary rainforest habitat over gravel and sand substrate with no aquatic vegetation and turbid water. The river is part of the middle course of the Kladuk River system by underground connection.

Physical water parameters at the type locality on May 2007 were: water temperature 28.0°C; pH 7.35; conductivity 368 μS. Chemical parameters (in mg/L): K: 0.1; Ca: 32.62; Mg: 2.55; Na: 0.29; Mn: 0.0; PO4: 0.1; SO4: 0.61; HCO3: 138; CO3: 0.0; Cl: 2.07; Cd: 0.0; Ni: 0.0.

Etymology

The species is named *longispina*, with reference from the Latin *longus* and *spinus*: named for the long spines on dorsal and anal fins.

Melanotaenia susii, sp. nov.
Kadarusman, Hubert & Pouyaud
(Figs 15, 16; Tab. IX)

Type material

Holotype. – MZB 22155, male, 114.1 mm SL, 1°09.114'S, 131°57.665'E, a bridge on Susi Creek, on the road from Sorong to Ajamaru, 12 km after the frontier post between Sorong and Sorong Selatan Regencies, Papua Barat Province, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 24 Apr. 2008

Paratypes. – MZB 22156, 4 specimens (94.5-111.7 mm SL), same data as for holotype; MZB 22157, 11 specimens (74.1-122.7 mm SL), collected at type locality by Kadarusman, Sudarto, Paradis & Pouyaud, 24 May 2007.

Table IX. - Measurements taken on the holotype and 15 paratypes of *Melanotaenia susii* sp. nov.

	Holotype	Paratypes				
SL (mm)	114.1	74.1-122.7				
in % standard length		n	Min	Max	Mean	SD
Head length	25.3	15	23.7	25.5	24.6	0.6
Snout length	10.2	15	9.2	9.9	9.4	0.2
Interorbital width	8.4	15	8.0	8.4	8.2	0.2
Eye diameter	6.6	15	6.4	7.3	6.8	0.3
Body depth	39.9	13	30.7	39.1	34.2	2.7
Body width	13.8	13	12.7	14.1	13.2	0.4
Caudal peduncle depth	10.8	15	9.8	10.8	10.4	0.4
Caudal peduncle length	14.8	14	13.9	15.3	14.6	0.5
Predorsal length	50.9	15	50.4	52.2	51.3	0.6
Prepelvic length	38.5	15	36.3	38.3	37.2	0.7
Preanal length	49.0	15	47.4	49.3	48.3	0.6
Pectoral fin length	18.3	15	17.6	19.0	18.3	0.5
Pelvic fin length	21.3	12	19.8	24.4	21.3	1.4
Spine length of first dorsal fin	12.4	13	11.4	12.4	11.8	0.4
Spine length of second dorsal fin		14	8.2	11.6	9.4	1.0
Spine length of anal fin	8.8	13	7.6	9.7	8.5	0.7
Dorsal fin base length	38.6	15	37.2	39.0	38.1	0.6
Second dorsal fin base length	25.7	15	23.9	26.6	25.3	0.9
Anal fin base length	44.6	15	41.1	45.6	43.1	1.4

Diagnosis

Melanotaenia susii is distinguished from all of its congeners in the Birds Head region by: dorsal rays IV to VI + I,13 to 16; anal rays I, 23 to 27; lateral scales 38 to 39; transverse scales 10 to 11; predorsal scales 17 to 20; cheek scales 16 to 20; total gillrakers on first arch 19; a compressed body with a long snout length, 9.2-10.2% of SL; a thin interorbital width, 8.0-8.4% of SL; a long predorsal length, 50.4-52.2% of SL; a short pectoral fin length, 17.6-19.0% of SL; a long pelvic fin length, 19.8-24.4% of SL; a long spine length on first dorsal fin, 11.4-12.4% of SL; a moderately long dorsal fin base length, 37.2-39.0% of SL; a long anal fin base length, 41.1-45.6% of SL; a marked red margin stripe on dorsal fins; 2-5 vertical bars extending across the entire body depth; dorsal fin origin vertically projected behind anal origin, between 3rd and 5th anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 15 specimens, 74.1-122.7 mm SL) if different from the holotype. Morphometric data are given in table IX.

Dorsal rays V + I,15 (IV-VI + I,13-16); anal rays I,27 (I,23-27); pectoral rays 14 (13-15); pelvic rays I,5; lateral scales 39 (38-39); transverse scales 10 (10-11); predorsal scales 19 (17-20); cheek scales 16 (16-20); total gillrakers on first arch 19; jaws equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips; teeth at front of upper jaw in about 6-7 irregular rows, reduced to 2 rows posteriorly; teeth at front of lower jaws in about 9-11 irregular rows, reduced to 2 rows posteriorly; small conical teeth on vomer and palatines.

Scale of body large, cycloid, crenulated, arranged in regular horizontal rows; predorsal scales extending to posterior margin of interorbital; 3 rows of preopercle scales. First dorsal fin origin projected behind anal origin, between 3rd and 5th anal soft ray; tip of first dorsal fin reaching base of 3rd to 5th soft ray of second dorsal fin; tip of second dorsal fin reaching ¾ of caudal peduncle in females and ¾ to hypural junction in males; tip of anal fin reaching 1/3 to half of caudal peduncle in females and half to ¾ of caudal peduncle in males; pelvic fin tips reaching base of 3rd to 7th anal soft ray; caudal fin moderately forked.

Colour of freshly collected male specimens: variable from green olive to intense black dorsally; lateral flanks with mauve reflections on upper half and reddish on lower half; a discontinuous midlateral dark green olive stripe with 1 scale row in middle part of body and 2 scale rows on caudal peduncle; 2-5 dark olive green vertical bars on anterior body region with

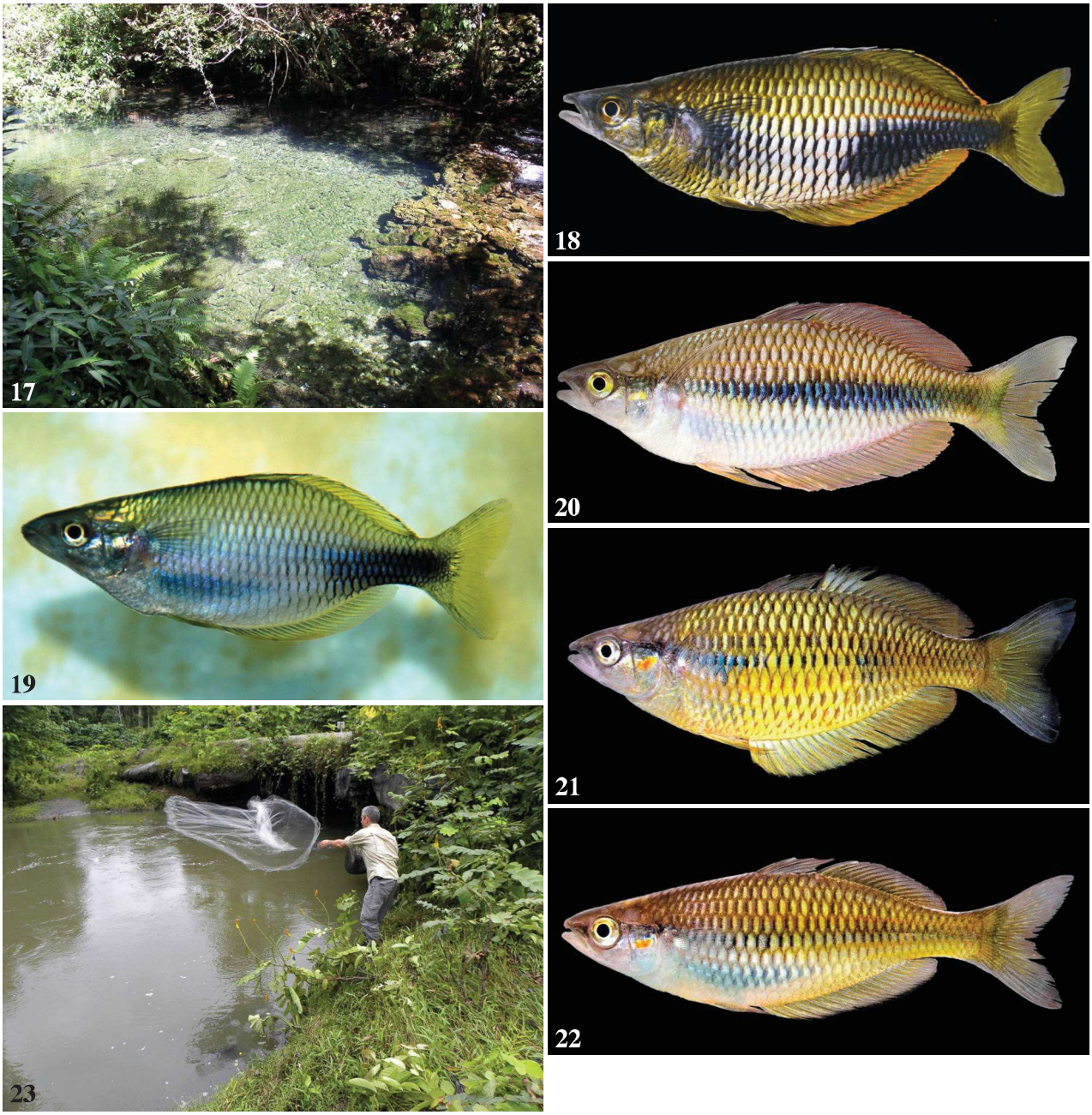

Figure 17. - Type locality of *Melanotaenia susii*, Susi Creek, Sorong Selatan, West Papua, Indonesia.
Figure 18. - *Melanotaenia sembrae*, MZB 22158 (holotype), male, 87.3 mm SL, Sembra River near Teminabuan, Sorong Selatan, West Papua, Indonesia.
Figure 19. - *Melanotaenia sembrae*, a female, 85 mm SL, same data as for holotype.
Figure 20. - *Melanotaenia manibuii*, MZB 22166 (holotype), male, 103.7 mm SL, Tisbo River near Bintuni, West Papua, Indonesia.
Figure 21. - *Melanotaenia naramasae*, MZB 22160 (holotype), male, 99.1 mm SL, Naramasa River, Birds Neck Isthmus
Figure 22. - *Melanotaenia naramasae*, MZB 22161 (paratype), female, 75.9 mm SL, same data as for holotype.
Figure 23. - Type locality of *Melanotaenia naramasae*, Naramasa River, Birds Neck, West Papua, Indonesia.

1-6 scales width and extending from dorsal to ventral edges; pectoral fin translucent with black rays; pelvic fin reddish black; anal fin overall dark with blue rays and with a reddish posterior tip; dorsal fins black with a marked red margin. Colour pattern of females similar, but less vivid than males.

Sexual dimorphism: males are slightly deeper bodied

than females (33.6-39.9 *vs* 31.0-37.0% SL) and have second dorsal and anal fins more elongated posteriorly.

Comparisons

Melanotaenia susii belongs to the "Central Ayamaru Plateau" group of species, together with *M. fasinensis*, *M. ericrobertsi*, *M. boesemani*, *M. ajamaruensis*, *M. klasioensis* sp. nov. and *M. longispina* sp. nov. (Fig. 2). The new species differs from all of these species by the genetic polymorphism assessed from the 12 microsatellite loci (Figs 3, 7).

Melanotaenia susii differs from *M. fasinensis* by the coloration pattern (presence of 2-5 vertical bars on anterior body region *vs* absent), more transversal scales (10-11 *vs* 9), a longer predorsal length (50.4-52.2 *vs* 48.1-49.8% SL), a longer pelvic fin length (19.8-24.4 *vs* 17.0-19.5% SL) and a shorter dorsal fin base length (37.2-39.0 *vs* 39.0-42.2% SL).

The new species differs from *M. ajamaruensis* and *M. boesemani* by a longer snout length (9.2-10.2 *vs* 7.2-8.7% SL), a longer pelvic fin length (19.8-24.4 *vs* 13.8-18.2% SL), a longer anal fin base length (41.1-45.6 *vs* 32.2-40.4% SL), more transverse scales (10-11 *vs* 7-8), more lateral scales (38-39 *vs* 33-37) and more predorsal scales (17-20 *vs* 15-16).

Following Allen *et al.* (2014b), *Melanotaenia susii* can be distinguished from *M. ericrobertsi* and *M. laticlavia* by the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 3rd and 5th anal soft ray *vs* dorsal fin origin about equal with anal fin origin), and the pattern of body coloration (presence of vertical bars on anterior body region *vs* absent). *Melanotaenia susii* differs from *M. laticlavia* by more lateral scales (38-39 *vs* 36-37) and more gillrakers (19 *vs* 16-17). It differs from *M. ericrobertsi* by more cheek scales (16-20 *vs* 12-16).

Melanotaenia susii differs from *M. longispina* sp. nov. by the presence of a dorsal margin stripe (*vs* absent), shorter spines on first dorsal, second dorsal and anal fins (11.4-12.4 *vs* 13.1-15.3, 8.2-11.6 *vs* 12.4-15.7, 7.6-9.7 *vs* 10.1-11.3% SL, respectively), a longer dorsal fin base length (37.2-39.0 *vs* 33.4-36.5% SL), and a longer second dorsal fin base length (23.9-26.6 *vs* 20.2-22.6% SL).

Melanotaenia susii differs from *M. klasioensis* sp. nov. by more lateral scales (38-39 *vs* 36-37), a longer snout length (9.2-10.2 *vs* 8.6-9.1% SL), a narrower interorbital width (8.0-8.4 *vs* 8.6-9.2% SL), a narrower body width (12.7-14.1 *vs* 14.2-15.8% SL), a longer predorsal length (50.4-52.2 *vs* 49.4-50.2% SL), a shorter pectoral fin length (17.6-19.0 *vs* 20.1-20.7% SL), and a longer spine length on first dorsal fin (11.4-12.4 *vs* 10.4-10.9% SL).

Distribution and habitat

The new species is currently known from Susi Creek, a karst resurgence of less than 3 m width with a course of a few kilometres before disappearing in a subterranean outlet (Fig. 1). The habitat at the type locality (Fig. 17) consists of clear, shallow and slow flowing water. The creek flows over limestone boulder and muddy bottom with many dead tree branches and is bordered by dense forest.

Physical water parameters at the type locality on May 2007 were: water temperature 26.0°C; pH 7.7; conductivity 330 µS. Chemical parameters (in mg/L): K: 0.01; Ca: 45.70; Mg: 2.17; Na: 0.27; Mn: 0.00; PO4: 0.09; SO4: 0.49; HCO3: 181; CO3: 0.0; Cl: 2.07; Cd: 0.0; Ni: 0.03.

Etymology

The species is named *susii*, with reference to the creek name at the type locality.

Melanotaenia sembrae, sp. nov.
Kadarusman, Carman & Pouyaud
(Figs 18, 19; Tab. X)

Type material

Holotype. – MZB 22158, male, 87.3 mm SL, 1°24.667'S, 131°59.231'E, Sembra River near Teminabuan, Sorong Selatan Regency, Papua Barat Province, Indonesia. Sumanta, Ajambua, Kadarusman, Slembrouck & Pouyaud, 23 Apr. 2008.

Paratypes. – MZB 22159, 9 specimens (69.5-101.7 mm SL), same data.

Diagnosis

Melanotaenia sembrae is distinguished from all of its congeners in the Birds Head region by the following combination of characters: dorsal rays IV to V + I,12 to 14; anal rays I,22 to 24; lateral scales 36 to 37; transverse scales 10; predorsal scales 17 to 19; cheek scales 12 to 15; total gillrakers on first arch 17 to 18; a pointed head with a long snout length, 9.1-9.7% of SL; an intermediate interorbital width, 8.4-8.8% of SL; a compressed body with a long predorsal length, 51.1-53.2% of SL; a great caudal peduncle depth, 10.7-11.6% of SL; a long pectoral fin length, 20.2-21.9% of SL; a moderately short dorsal fin base length, 32.9-38.5; a marked red margin stripe on second dorsal fin; dorsal fin origin vertically projected behind anal origin, between 3rd and 5th anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 9 specimens, 69.5-101.7 mm SL) if different from the holotype. Morphometric data are given in table X.

Dorsal rays V + I,13 (IV-V + I,12-14); anal rays I,23 (I,22-24); pectoral rays 16 (14-16); pelvic rays I,5; lateral scales 36 (36-37); transverse scales 10; predorsal scales 19 (17-19); cheek scales 15 (12-14); total gillrakers on first

arch 18 (17-18); jaws equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends behind anterior edge of eye; lips thin; teeth conical with slightly curved tips; teeth at front of upper jaw in about 4-6 irregular rows, reduced to 1-2 rows posteriorly; teeth at front of lower jaws in about 6-8 irregular rows, reduced to 1-2 rows posteriorly; small conical teeth on vomer and palatines.

Scale of body large, cycloid, crenulated, arranged in regular horizontal rows; predorsal scales extending to posterior margin of interorbital; 3 rows of preopercle scales. First dorsal fin origin projected behind anal origin, between 3rd and 5th anal soft ray; tip of first dorsal fin reaching base of 2nd to 4th soft ray of second dorsal fin in males and spine to 1st soft ray in females; tip of second dorsal fin reaching half of caudal peduncle in females and ¾ in males; tip of anal fin reaching 1/3 to half of caudal peduncle in females and half to ¾ of caudal peduncle in males; pelvic fin tips reaching base of 1st to 4th anal soft ray; caudal fin forked.

Colour of freshly collected male specimens: overall body green olive; posterior part of lateral flanks with 6-7 narrow orange-red stripes alternating with horizontal scale rows; a discontinuous midlateral stripe of 3-4 scales wide, varying from intense black to blue steel and marked on caudal peduncle and just behind pectoral fins; pectoral fin translucent; anal fin green olive with orange-red hues; remaining fins green olive with marked orange-red margin on second dorsal fin. Overall colour of female paler, with absence of orange hues on the anal fin and between horizontal scales rows.

Sexual dimorphism: males are deeper bodied than females (31.5-37.2 *vs* 31.2% SL) and have the two dorsals and anal fins more elongated posteriorly.

Table X. - Measurements taken on the holotype and nine paratypes of *Melanotaenia sembrae* sp. nov.

	Holotype	Paratypes			
SL (mm)	87.3	69.5-101.7			
in % standard length		n	Min	Max	Mean	SD
Head length	25.4	9	24.6	26.6	25.4	0.8
Snout length	9.4	9	9.1	9.7	9.3	0.3
Interorbital width	8.4	9	8.5	8.8	8.6	0.1
Eye diameter	7.2	9	6.9	7.9	7.5	0.4
Body depth	34.6	9	31.2	37.2	33.7	2.2
Body width	14.0	9	12.9	14.3	13.5	0.5
Caudal peduncle depth	10.8	9	10.7	11.6	11.0	0.3
Caudal peduncle length	14.8	9	14.0	16.2	14.8	0.8
Predorsal length	52.0	9	51.1	53.2	52.0	0.7
Prepelvic length	37.3	9	37.3	39.4	38.5	0.7
Preanal length	48.6	9	48.5	52.7	50.8	1.3
Pectoral fin length	20.4	9	20.2	21.9	20.9	0.6
Pelvic fin length	17.2	9	15.9	21.3	19.1	1.9
Spine length of first dorsal fin	9.7	9	9.7	11.1	10.5	0.6
Spine length of second dorsal fin	8.3	9	7.7	9.3	8.6	0.5
Spine length of anal fin	8.2	9	7.4	9.8	8.5	0.8
Dorsal fin base length	37.1	9	32.9	38.5	36.5	2.1
Second dorsal fin base length	23.7	9	20.5	24.8	23.2	1.4
Anal fin base length	43.6	9	35.3	43.1	39.8	2.3

Comparisons

Melanotaenia sembrae is genetically allied to *M. multiradiata* in the COI phylogeny (Fig. 2). Both species belong to the "Southern Ayamaru" species cluster which is the sister group of the "Central Ayamaru Plateau" cluster and of the monospecific cluster represented by *M. arfakensis*. The new species is easily distinguishable from all these species based on its microsatellite genotype diversity (Figs 4, 7).

Melanotaenia sembrae differs from *M. multiradiata* by the overall body form with a greater interorbital width (8.4-8.8 *vs* 7.7-8.3% SL), a greater body depth (31.2-37.2 *vs* 27.1-30.5% SL), a greater body width (12.9-14.3 *vs* 11.2-12.6% SL), a greater caudal peduncle depth (10.7-11.6 *vs* 9.3-10.0% SL), a longer predorsal length (51.1-53.2 *vs* 49.5-50.9% SL), a longer pectoral fin length (20.2-21.9 *vs* 16.9-19.2% SL), a shorter spine length of second dorsal fin (7.7-9.3 *vs* 10.0-13.6% SL), fewer anal fin rays (22-24 *vs* 24-27), and fewer total gillrakers on first arch (17-18 *vs* 18-19).

Melanotaenia sembrae differs from *M. arfakensis* by a longer snout length (9.1-9.7 *vs* 7.6-8.2% SL), a slender interorbital width (8.4-8.8 *vs* 8.8-9.3% SL), a shorter and slender caudal peduncle (its length 14.0-16.2 *vs* 16.5-18.3 and depth 10.7-11.6 *vs* 12.0-13.2% SL), a longer predorsal length (51.1-53.2 *vs* 47.1-49.6% SL), more lateral scales (36-37 *vs* 34-36), and more gillrakers (17-18 *vs* 15-16).

The new species differs from *M. ajamaruensis* and *M. boesemani* by more transverse scales (10 *vs* 7-8), more predorsal scales (17-19 *vs* 15-16), a longer snout length (9.1-9.7 *vs* 7.2-8.7% SL) and a longer pectoral fin length (20.2-21.9 *vs* 16.9-19.9% SL).

Melanotaenia sembrae is distinguishable from *M. fasinensis* by more transverse scales (10-12 *vs* 9), fewer dorsal soft rays (12-14 *vs* 14-17), fewer anal rays (22-24 *vs* 24-27), fewer lateral scales (36-37 *vs* 37-39), a greater interorbital width (8.4-8.8 *vs* 7.6-8.2% SL), a longer predorsal length (51.1-53.2 *vs* 48.1-49.8% SL), and several other characters (see morphometric data related to *M. fasinensis* in appendix).

Melanotaenia sembrae differs from *M. ericrobertsi* and *M. laticlavia* (see Allen *et al.*, 2014b) by the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 3rd and 5th anal soft ray *vs* dorsal fin origin about equal with anal fin origin). It also differs from *M. ericrobertsi* by fewer lateral scales (36.37 *vs*

37-39) and from *M. laticlavia* by fewer dorsal rays (12-14 *vs* 14-17), fewer anal rays (22-24 *vs* 24-26), more gillrakers (17-18 *vs* 16-17).

Melanotaenia sembrae differs from *M. klasioensis* sp. nov. by a longer predorsal length (51.1-53.2 *vs* 49.4-50.2% SL), a shorter dorsal fin base length (32.9-38.5 *vs* 38.7-40.3% SL), fewer dorsal soft rays (12-14 *vs* 14-15), fewer cheek scales (12-15 *vs* 16-17) and fewer gillrakers (17-18 *vs* 18-20). It differs from *M. longispina* sp. nov. by the general pattern of coloration, the presence of a dorsal margin stripe (*vs* absent), fewer lateral scales (36-37 *vs* 37-40), fewer predorsal scales (17-19 *vs* 19-21), fewer cheek scales (12-15 *vs* 17-18), fewer gillrakers (17-18 *vs* 19) and several other morphological characters (see Tab. VIII for comparisons). *Melanotaenia sembrae* differs from *M. susii* sp. nov. by fewer lateral scales (36-37 *vs* 38-39), fewer cheek scales (12-15 *vs* 16-20), fewer gillrakers (17-18 *vs* 19), a greater interorbital width (8.4-8.8 *vs* 8.0-8.4% SL), a longer pectoral fin length (20.2-21.9 *vs* 17.6-19.0% SL), a shorter spine length on first dorsal fin (9.7-11.1 *vs* 11.4-12.4% SL), and the coloration pattern (no vertical bars *vs* presence of 3-5 vertical bars on anterior body region).

Distribution and habitat

The new species is currently known from the Sembra River, a karst resurgence emerging from the southern side of the Ayamaru Plateau, west of Teminabuan town and flowing into Teminabuan Bay (Fig. 1). The type locality consists of a 20-25 m wide stream with depths to about 4 m and moderate to fast flow through second growth forest. The specimens were collected over a limestone rock bottom in clear water.

Etymology

The species is named *sembrae*, with reference to the river name at the type locality.

Melanotaenia manibuii, sp. nov.
Kadarusman, Slembrouck & Pouyaud
(Fig. 20; Tab. XI)

Type material

Holotype. – MZB 22166, male, 103.7 mm SL, 1°56.330'S, 133°31.036'E, Tisbo R. near Bintuni, Papua Barat Province, Indonesia. Sumanta, Ajambua, Kadarusman, 4 Jun. 2007.

Paratypes. – MZB 22167, 14 specimens (66.4-106.7 mm SL), same data.

Diagnosis

Melanotaenia manibuii is distinguished from all of its congeners in the Birds Head region by the following combination of characters: dorsal rays IV to V + I,13 to 16; anal rays I, 22 to 25; lateral scales 35 to 37; transverse scales 10; predorsal scales 15 to 18; cheek scales 13 to 19; gillrakers 18 to 19; a short head length, 24.0-25.1% of SL; a long snout length, 8.6-9.3% of SL; a broad interorbital width, 8.8-9.2% of SL; a broad caudal peduncle depth, 11.7-13.3% of SL; a short caudal peduncle length, 13.6-15.4% of SL; a short predorsal length, 47.5-49.9% of SL; a moderately short spine length on first dorsal fin, 9.7-12.0% of SL; an intermediate spine length on second dorsal fin, 7.8-12.2% of SL, and on the anal fin, 7.2-9.8% of SL; moderately long dorsal fin base length, 37.2-41.8% of SL, and second dorsal fin base length, 24.0-28.7% of SL; a moderately long anal fin base length, 40.0-43.9% of SL; no stripe on the margin of the dorsal and anal fins; dorsal fin origin vertically projected behind anal origin, between 1st and 3rd anal soft ray; absence of sexual dimorphism in contrast to most members of the genus.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 14 specimens, 66.4-106.7 mm SL) if different from the holotype. Morphometric data are given in table XI.

Dorsal rays V + I,14 (IV-V + I,13-16); anal rays I,24 (I,22-25); pectoral rays 14 (14-15); pelvic rays I,5; lateral scales 37 (35-37); transverse scales 10; predorsal scales 16 (15-18); cheek scales 19 (13-19); total gillrakers on first arch 18 (18-19); jaws equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips; teeth at front of upper jaw in about 5-7 irregular rows, reduced to 1-2 rows posteriorly; teeth at front of lower jaws in about 6-10 irregular rows, reduced to 1-2 rows posteriorly; small conical teeth on vomer and palatines.

Scale of body large, cycloid, crenulated, arranged in regular horizontal rows; predorsal scales extending to posterior margin of interorbital; 3 rows of preopercle scales. First dorsal fin origin projected behind anal origin, between 1st and 3rd anal soft ray; tip of first dorsal fin reaching base of spine or 1st to 4th soft ray of second dorsal fin; tip of second dorsal fin reaching middle of caudal peduncle; pelvic fin tips reaching base of 4th to 5th anal soft ray; caudal fin forked.

Colour of freshly collected male specimens: upper half of the body, nape and dorsal region brown with olive green reflections; lower half of the body and belly whitish; an intense and continuous black (sometimes with blue hues) midlateral stripe of 1 scale wide on pectoral region and 2-3 scales wide on caudal peduncle; pectoral fin translucent; anal and dorsal fins uniformly pale red; a brownish caudal fin with green hues at its base and extending to caudal peduncle; pelvic fins yellowish; a diffuse blue blotch on side of abdomen. Colour pattern of females similar, but paler than males. No sexual dimorphism.

Comparisons

Melanotaenia manibuii and *M. irianjaya* are geographically close (approx. 90 km between both type localities separated by the Bintuni Bay) and possess similar meristic features (see appendix). Nevertheless, both species belong to distinct phylogenetic groups with important genetic divergence (Fig. 2). *Melanotaenia manibuii* belongs to the "Northern Birds Head" group, while *M. irianjaya* belongs to the "Southern Birds Head" group. *Melanotaenia manibuii* is easily distinguishable from *M. irianjaya* by a shorter head length (24.0-25.1 *vs* 25.3-27.0% SL), a greater caudal peduncle depth (11.7-13.3 *vs* 9.7-11.1% SL), a shorter predorsal length (47.5-49.9 *vs* 50.0-52.5% SL), shorter spine length of first and second dorsal fin (respectively 9.7-12.0 *vs* 12.3-13.6 and 7.8-12.2 *vs* 12.3-14.1% SL), a shorter spine length of anal fin (7.2-9.8 *vs* 9.8-11.6% SL), a longer second dorsal fin base length (24.0-28.7 *vs* 21.1-23.7% SL), fewer gillrakers (18-19 *vs* 19-21), and by the absence of a stripe on the dorsal fin margin.

The new species is easily distinguishable from all of the species belonging to the "Northern Birds Head" group by the genetic polymorphism assessed from the 12 microsatellite loci (Figs 5, 6, 7) and by the morphological characters given below.

Melanotaenia manibuii differs from *M. misoolensis* by a shorter head length (24.0-25.1 *vs* 25.4-26.6% SL), a smaller eye diameter (6.8-8.0 *vs* 8.4-9.9% SL), a deeper and shorter caudal peduncle (its depth 11.7-13.3 *vs* 10.7-11.3 and length 13.6-15.4 *vs* 15.6-16.5% SL). It differs from *M. flavipinnis* by the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 3rd anal soft ray *vs* dorsal fin origin between 5th and 6th anal fin origin) and by the general pattern of coloration (see Allen *et al.*, 2014a for more details). The new species differs from *M. batanta* and *M. salawati* by a shorter predorsal length (47.5-49.9 *vs* 50.8-54.8% SL) and a shorter anal fin base length (40.0-43.9 *vs* 44.0-50.5% SL). Compared to *M. fredericki*, the new species displays a longer snout length (8.6-9.3 *vs* 7.7-8.4% SL), a greater interorbital width (8.8-9.2 *vs* 8.0-8.4% SL) and a greater caudal peduncle depth (11.7-13.3 *vs* 9.3-11.0% SL). The new species differs from *M. arfakensis* by a longer snout length (8.6-9.3 *vs* 7.6-8.2% SL) and a shorter caudal peduncle length (13.6-15.4 *vs* 16.5-18.3% SL).

Many characters enable *M. manibuii* to be distinguished from *M. longispina* sp. nov, such as a greater interorbital width (8.8-9.2 *vs* 7.8-8.2% SL), a broader caudal peduncle (its depth 11.7-13.3 *vs* 9.2-10.7 and length 13.6-15.4 *vs* 15.4-16.7% SL), a shorter predorsal length (47.5-49.9 *vs* 50.2-51.8% SL), shorter spine length on first dorsal (9.7-12.0 *vs* 13.1-15.3) and second dorsal fins (7.8-12.2 *vs* 12.4-15.7% SL), a shorter spine length of anal fin (7.2-9.8 *vs* 10.1-11.3% SL), a longer total dorsal fin base (37.2-41.8 *vs* 33.4-36.5) and second dorsal fin base lengths (24.0-28.7 *vs* 20.2-22.6% SL).

Following Allen *et al.* (2014b), *M. manibuii* differs from *M. laticlavia* and *M. ericrobertsi* by the pattern of coloration of the dorsal fin (absence of margin stripe *vs* present) and the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 3rd anal soft ray *vs* dorsal fin origin about level). The new species also displays more gillrakers than *M. laticlavia* (18-19 *vs* 16-17) and fewer lateral scales than *M. ericrobertsi* (35-37 *vs* 37-39).

Melanotaenia manibuii differs from the remaining members of the "Central Ayamaru Plateau" and the "Southern Ayamaru Plateau" species clusters by the pattern of coloration of the dorsal fin (absence of margin stripe *vs* present) and several other morphological characters (see Tabs VII, IX, X and Appendix for more details).

Table XI. - Measurements taken on the holotype and 14 paratypes of *Melanotaenia manibuii* sp. nov.

	Holotype	Paratypes				
SL (mm)	103.7	66.4-106.7				
in % standard length		n	Min	Max	Mean	SD
Head length	25.0	14	24.0	25.1	24.8	0.3
Snout length	9.3	14	8.6	9.2	9.0	0.2
Interorbital width	8.8	14	8.8	9.2	8.9	0.1
Eye diameter	7.0	14	6.8	8.0	7.3	0.3
Body depth	35.3	14	32.3	38.2	33.8	1.6
Body width	14.2	14	13.5	15.4	14.0	0.6
Caudal peduncle depth	12.3	13	11.7	13.3	12.3	0.5
Caudal peduncle length	15.3	14	13.6	15.4	14.7	0.5
Predorsal length	48.4	14	47.5	49.9	48.8	0.8
Prepelvic length	36.7	14	36.7	39.0	37.8	0.7
Preanal length	47.2	14	48.1	51.2	49.2	0.8
Pectoral fin length	19.2	14	17.4	20.4	19.1	0.9
Pelvic fin length	21.9	14	19.4	22.7	21.1	1.1
Spine length of first dorsal fin	11.3	14	9.7	12.0	11.1	0.6
Spine length of second dorsal fin	9.6	14	7.8	12.2	10.5	1.1
Spine length of anal fin	9.2	14	7.2	9.8	8.9	0.8
Dorsal fin base length	40.3	14	37.2	41.8	39.2	1.3
Second dorsal fin base length	27.7	14	24.0	28.7	25.8	1.5
Anal fin base length	43.7	14	40.0	43.9	41.9	1.3
Anal fin height	16.1	4	13.4	16.8	14.6	1.5

Distribution and habitat

The new species is currently known from the Tisbo River in the southern foothills of the Arfak Mountains at an elevation of about 87 m, approximately 20 km north of Bintuni town. The Tisbo River is part of the Tembuni River system, which

flows into Bintuni Bay on the south coast of the Birds Head Peninsula. The stream, which ranged between 5 and 10 m in width at the type locality, was flowing through secondary forest habitat over gravel and sand substrate.

Etymology

The species is named *manibuii*, in honour of Alfons Manibui, Bupati from Bintuni.

***Melanotaenia naramasae*, sp. nov.**
Kadarusman, Nugraha & Pouyaud
(Figs 21, 22; Tab. XII)

Type material

Holotype. – MZB 22160, male, 99.1 mm SL, 2°48.440'S, 134°19.965'E, Naramasa R., Birds Neck Isthmus, Papua Barat province, Indonesia. Kadarusman, Ogistira, Sumanta & Pouyaud, 21 Apr. 2009.

Paratypes. – MZB 22161, 7 specimens (75.9-97.3 mm SL), same data.

Diagnosis

Melanotaenia naramasae is distinguished from all of its congeners in the Birds Head region by the following combination of characters: dorsal rays IV to V + I,13 to 15; anal rays I, 21 to 24; lateral scales 33 to 35; transverse scales 10; predorsal scales 16 to 17; cheek scales 13 to 16; gillrakers 15; a large body depth, 33.3-37.8, with a moderately short prepelvic length 36.3-38.3% of SL; a moderately short caudal peduncle length, 12.9-16.3, with a large depth, 11.8-12.4% of SL; a moderately long pectoral fin length, 18.7-21.7, and a long pelvic fin length 19.4-23.5% of SL; a long dorsal fin base length, 38.4-41.4% of SL; a moderately long anal fin base length, 39.8-46.0; no stripe on the anal and dorsal fin margins; dorsal origin behind anal origin between 1st and 2nd anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 7 specimens, 75.9-97.3 mm SL) if different from the holotype. Morphometric data are given in table XII.

Dorsal rays V + I,15 (IV-VI + I,13-15); anal rays I,24 (I,21-24); pectoral rays 14 (13-14); pelvic rays I,5; lateral scales 34 (34-35); transverse scales 10; predorsal scales 16 (16-17); cheek scales 16 (13-16); total gillrakers on first arch 15; head rounded; jaws about equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips extending to outer surface of lips; teeth of upper jaws in about 6-7 irregular rows, reduced to 1-2 rows posteriorly; lower jaws in about 5-8 irregular rows anteriorly, reduced to 1-2 rows posteriorly; vomer and palatines edentate.

Scale of body cycloid, relatively large, and arranged in regular horizontal rows; scale margin slightly crenulate; predorsal scales extending to middle portion of interorbital; preopercle scales from posterior angle to edge of eye arranged in 3 rows.

First dorsal fin origin behind anal fin origin, between 1st to 2nd anal soft rays; tip of first dorsal fin reaching 2nd to 3rd soft ray of second dorsal fin; tip of second dorsal fin reaching hypural junction for the males and half length of caudal peduncle for the females; pelvic fin tips when depressed reaching base of 2nd to 3rd soft anal fin ray for females and 3rd to 6th for males; caudal fin moderately forked.

Colour of freshly collected male specimens: overall yellow; nape and dorsal part of head brown, belly yellowish; a dark blue midlateral stripe of 1-2 scale wide and not continuous, intense with 2 scales wide just behind pectoral fin base, interrupted on middle of side and faint on caudal peduncle; pectoral fin translucent; remaining fins yellow; no margin stripe on dorsal and anal fins; colour pattern of females similar but less vivid on fins and on lower part of the body with presence of a large blue blotch extending from pectoral fin base to middle of anal fin.

Table XII. - Measurements taken on the holotype and seven paratypes of *Melanotaenia naramasae* sp. nov.

	Holotype	Paratypes			
SL (mm)	99.1	75.9-97.3			
in % standard length		n	Min	Max	Mean	SD
Head length	24.7	7	24.9	26.2	25.5	0.4
Snout length	8.8	7	8.6	9.5	9.0	0.3
Interorbital width	8.4	7	8.4	9.0	8.7	0.2
Eye diameter	7.5	7	7.1	8.1	7.6	0.3
Body depth	36.4	7	33.3	37.8	35.1	1.6
Body width	13.7	7	12.5	14.4	13.7	0.6
Caudal peduncle depth	11.8	7	11.9	12.4	12.1	0.2
Caudal peduncle length	14.3	7	12.9	16.3	15.3	1.2
Predorsal length	48.9	7	48.4	51.4	49.5	1.0
Prepelvic length	37.0	7	36.3	38.3	37.4	0.7
Preanal length	49.2	7	46.8	51.4	49.1	1.6
Pectoral fin length	19.3	6	18.7	21.7	20.1	1.0
Pelvic fin length	20.0	6	19.4	23.5	21.3	1.5
Spine length of first dorsal fin	9.1	7	9.0	11.0	10.3	0.7
Spine length of second dorsal fin	7.9	7	7.3	10.9	9.2	1.3
Spine length of anal fin	6.8	7	7.4	9.2	8.7	0.6
Dorsal fin base length	40.5	7	38.4	41.4	39.5	1.1
Second dorsal fin base length	26.8	7	24.0	26.4	25.5	0.9
Anal fin base length	44.2	7	39.8	46.0	42.0	2.4
Anal fin height		3	13.0	16.5	14.6	1.7

Sexual dimorphism: Males are deeper bodied than females (body depth 35.4-37.8 *vs* 33.3-35.3% SL) and have a longer second dorsal fin base (25.9-26.8 *vs* 24.0-26.0% SL).

Comparisons

Melanotaenia naramasae is part of the "Southern Birds Head" group of species and belongs to the "Birds Neck" species cluster together with *M. angfa* and *M. parva* (see Fig. 2). The new species is also easily distinguishable from these species on the basis of the polymorphism of the 12 analyzed microsatellite loci (Fig. 8).

Melanotaenia naramasae differs from *M. angfa* by a greater body depth (33.3-37.8 *vs* 28.8-32.3% SL), a greater caudal peduncle depth (11.8-12.4 *vs* 10.2-11.4% SL), a longer pectoral fin length (18.7-21.7 *vs* 16.2-18.5% SL), fewer lateral scales (33-35 *vs* 35-36), fewer gillrakers (15 *vs* 16-20), and the position of the first dorsal fin origin compared to the anal fin origin (dorsal fin origin behind anal origin, between 1st and 2nd anal soft ray *vs* dorsal fin origin before anal fin origin). It differs from *M. parva* by a shorter caudal peduncle length (12.9-16.3 *vs* 16.8-19.4% SL), a shorter prepelvic length (36.3-38.3 *vs* 38.3-41.0% SL), a longer pelvic fin length (19.4-23.5 *vs* 15.1-17.3% SL), a longer dorsal fin base length (38.4-41.4 *vs* 33.8-37.4% SL), a longer anal fin base length (39.8-46.0 *vs* 33.1-38.2% SL), more dorsal soft rays (13-15 *vs* 11-13), more cheek scales (13-16 *vs* 10-13), and position of first dorsal fin origin relatively to anal fin origin (behind *vs* before).

Distribution and habitat

The new species is currently known only from the upstream part of the Naramasa River system flowing westward to Bintuni Bay. The stream, which ranged between 20 and 30 m in width at the type locality, was flowing through primary forest habitat over gravel and sand substrate. Rainbowfishes were mainly concentrated along the riverbank around log debris.

Etymology

The species is named *naramasae*, in reference to the river name at the type locality.

Table XIII. - Measurements taken on the holotype and 11 paratypes of *Melanotaenia rumberponensis* sp. nov.

	Holotype	Paratypes				
SL (mm)	65.2	50.2-70.0				
in % standard length		n	Min	Max	Mean	SD
Head length	26.3	11	26.2	27.4	26.8	0.3
Snout length	8.4	11	8.2	8.7	8.4	0.2
Interorbital width	9.2	11	9.1	9.5	9.3	0.2
Eye diameter	8.9	11	8.7	9.3	9.0	0.2
Body depth	31.6	11	29.9	33.2	31.6	1.1
Body width	13.3	11	12.7	14.5	13.7	0.6
Caudal peduncle depth	11.0	11	10.3	11.1	10.7	0.3
Caudal peduncle length	13.1	11	12.6	14.4	13.7	0.6
Predorsal length	50.3	11	48.4	50.3	49.4	0.6
Prepelvic length	39.2	11	39.0	40.4	39.8	0.5
Preanal length	50.1	11	49.7	52.5	50.9	0.9
Pectoral fin length	19.6	11	17.5	19.6	18.7	0.7
Pelvic fin length	17.1	11	15.1	17.9	16.8	0.8
Spine length of first dorsal fin	11.5	11	10.7	12.7	11.8	0.7
Spine length of second dorsal fin	10.4	11	8.1	12.8	10.2	1.3
Spine length of anal fin	8.7	11	7.7	9.0	8.3	0.4
Dorsal fin base length	41.6	11	37.5	42.7	40.2	1.8
Second dorsal fin base length	25.6	11	22.2	26.8	24.7	1.6
Anal fin base length	43.7	11	36.3	43.0	39.7	2.3
Anal fin height	14.0	3	11.7	13.6	12.7	0.9

Melanotaenia rumberponensis, sp. nov.
Kadarusman, Ogistira & Pouyaud
(Fig. 24; Tab. XIII)

Type material

Holotype. – MZB 22162, male, 65.2 mm SL, 1°51.238'S, 134°09.489'E, Rumberpon Island, Cendrawasih Bay, Birds Neck Isthmus, Papua Barat Province, Indonesia. Kadarusman, Ogistira, Ajambua, Sumanta & Pouyaud, 5 Apr. 2008.

Paratypes. – MZB 22163, 11 specimens (50.2-70.0 mm SL), same data as holotype.

Diagnosis

Melanotaenia rumberponensis is distinguished from all of its congeners in the Birds Head region by: dorsal rays IV to V + I,13 to 14; anal rays I, 21 to 23; lateral scales 34 to 35; transverse scales 10-11; predorsal scales 16 to 18; cheek scales 14 to 16; gillrakers 18; a short snout length, 8.2-8.7% of SL; a broad interorbital width, 9.1-9.5% of SL; a large eye diameter, 8.7-9.3% of SL; an elongate body, its depth 29.9-33.2; a long prepelvic length, 39.0-40.4% of SL; a moderately short predorsal length, 48.4-50.3% of SL; a caudal peduncle short and slender, its length 12.6-14.4 and its depth 10.3-11.1% of SL; a short pelvic fin length, 15.1-17.9% of SL; a moderately long dorsal fin base length, 37.5-42.7% of SL; no stripe on the margin of the anal and dorsal fins; a continuous black midlateral stripe, covering 3 scales wide from upper edge of preopercle to caudal fin base; dorsal origin aligned with anal origin or behind it, until the 3rd anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 11

Figure 24. - *Melanotaenia rumberponensis*, MZB 22162 (holotype), male, 65.2 mm SL, Rumberpon Island, Cendrawasih Bay, Birds Neck, West Papua, Indonesia.
Figure 25. - *Melanotaenia sikuensis*, MZB 22164 (holotype), male, 91.0 mm SL, Siku Creek, Birds Neck, West Papua, Indonesia.
Figure 26. - *Melanotaenia sikuensis*, MZB 22165 (paratype), female, 77.6 mm SL, same data as for holotype.

specimens, 50.2-70.0 mm SL) if different from the holotype. Morphometric data are given in table XIII.

Dorsal rays V + I,14 (IV-V + I,13-14); anal rays I,22 (I,21-23); pectoral rays 14 (13-14); pelvic rays I,5; lateral scales 35 (34-35); transverse scales 11 (10-11); predorsal scales 17 (16-18); cheek scales 14 (14-16); total gillrakers on first arch 18; body elongated with head and eyes well developed; jaws about equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips extending to outer surface of lips; teeth of upper jaws in about 3-5 irregular rows, reduced to 1-2 rows posteriorly; lower jaws in about 4-5 irregular rows anteriorly, reduced to 1-2 rows posteriorly; several small conical teeth on vomer and palatines.

Scale of body cycloid, relatively large, and arranged in regular horizontal rows; scale margin slightly crenulate; predorsal scales extending to posterior portion of interorbital; preopercle scales from posterior angle to edge of eye arranged in 3 rows.

First dorsal fin origin vertically aligned with anal origin or behind it, until the 3rd anal soft ray; tip of first dorsal fin reaching 2nd to 3rd soft ray of second dorsal fin for males and reaching spine of second dorsal fin for females; tip of second dorsal fin reaching 2/3 length of caudal peduncle for the males and half length for the females; pelvic fin tips when depressed reaching base of 1st to 2nd soft anal fin ray for females and 2nd to 3rd for males; caudal fin forked.

Colour of freshly collected male specimens: overall yellow; dorsal part of head and body brownish, belly yellowish; a continuous black midlateral stripe, covering 3 scales wide from upper edge of preopercle to caudal fin base; pectoral fin translucent; remaining fins yellow; no margin stripe on dorsal and anal fins; a faint blue blotch extending from pectoral fin base to middle of anal fin; colour of females with equal intensity.

Sexual dimorphism: Males have longer fins than females, are generally deeper bodied (31.6-33.2 *vs* 29.9-32.1% SL) and have a deeper caudal peduncle (10.7-11.1 *vs* 10.3-10.9% SL).

Comparisons

Melanotaenia rumberponensis is part of the "Southern Birds Head" group of species and belongs to the "Birds Neck" species cluster together with *M. angfa*, *M. parva* and *M. naramasae* sp. nov. (Fig. 2). The new species is distinguishable from all of these species on the basis of the polymorphism of the 12 analyzed microsatellite loci (Fig. 8).

Melanotaenia rumberponensis is distinguished from *M. angfa* and *M. parva* by a greater eye diameter (8.7-9.3 *vs* 7.3-8.4), a shorter caudal peduncle length (12.6-14.4 *vs* 14.6-19.4% SL) and by the thickness of the midlateral stripe on the caudal peduncle region (3 scales wide *vs* faint or absent). In addition, the new species differs from *M. angfa* by a shorter snout length (8.2-8.7 *vs* 9.0-9.5% SL), fewer lateral scales (34-35 *vs* 35-36), and fewer soft dorsal rays (13-14 *vs* 14-16). The new species differs from *M. parva* by a slender caudal peduncle depth (10.3-11.1 *vs* 11.6-12.5% SL), a shorter predorsal length (48.4-50.3 *vs* 50.8-52.8% SL), a longer dorsal fin base length (37.5-42.7 *vs* 33.8-37.4% SL), more cheek scales (14-16 *vs* 10-13) and more gillrakers (18 *vs* 13-16).

Melanotaenia rumberponensis differs from *M. naramasae* sp. nov. by a greater interorbital width (9.1-9.5 *vs* 8.4-9.0% SL), a greater eye diameter (8.7-9.3 *vs* 7.1-8.1% SL), a slender body depth (29.9-33.2 *vs* 33.3-37.8) and caudal

peduncle depth (10.3-11.1 *vs* 11.8-12.4% SL), a longer prepelvic length (39.0-40.4 *vs* 36.3-38.3% SL), a shorter pelvic fin length (15.1-17.9 *vs* 19.4-23.5% SL) and more gillrakers (18 *vs* 15).

Distribution and habitat

The new species is currently known from the type locality, a swamp drained by a small creek of 5 m in width flowing in the central part of the island. The stream was flowing through primary forest habitat over muddy substrate with abundant aquatic vegetation.

Etymology

The species is named *rumberponensis*, in reference to the type locality, the Rumberpon Island.

***Melanotaenia sikuensis*, sp. nov.**
Kadarusman, Sudarto & Pouyaud
(Figs 25, 26; Tab. XIV)

Type material

Holotype. – MZB 22164, male, 91.0 mm SL, 1°46.784'S, 134°04.790'E, Siku Creek, Birds Neck Isthmus, Papua Barat Province, Indonesia. Kadarusman, Paradis, Ajambua, Sumanta & Pouyaud, 2 Jun. 2007.

Table XIV. - Measurements taken on the holotype and 10 paratypes of *Melanotaenia sikuensis* sp. nov.

	Holotype	Paratypes				
SL (mm)	91.0	63.9-81.7				
in % standard length		n	Min	Max	Mean	SD
Head length	24.7	10	25.3	26.1	25.8	0.2
Snout length	8.2	10	8.1	8.9	8.6	0.3
Interorbital width	8.2	10	8.3	8.7	8.6	0.2
Eye diameter	7.1	10	7.3	8.0	7.7	0.2
Body depth	34.8	10	30.1	35.6	33.2	1.7
Body width	13.7	10	12.6	15.0	13.4	0.9
Caudal peduncle depth	11.2	10	10.1	11.5	11.0	0.4
Caudal peduncle length	13.9	10	12.9	14.3	13.8	0.6
Predorsal length	48.6	10	47.7	49.5	48.8	0.6
Prepelvic length	37.3	10	36.7	39.0	38.0	0.7
Preanal length	48.4	10	47.2	50.6	49.3	1.0
Pectoral fin length		10	18.6	20.7	19.4	0.6
Pelvic fin length	18.1	10	15.5	19.3	17.6	1.1
Spine length of first dorsal fin	8.8	10	9.9	11.5	10.4	0.6
Spine length of second dorsal fin	6.5	10	7.6	8.7	8.1	0.5
Spine length of anal fin	6.3	10	6.2	9.5	7.2	1.0
Dorsal fin base length	41.9	10	38.5	42.1	39.6	1.2
Second dorsal fin base length	28.4	10	24.7	28.0	26.3	1.1
Anal fin base length	42.8	10	39.5	43.1	41.0	1.4
Anal fin height		3	14.4	15.7	15.1	0.6

Paratypes. – MZB 22165, 10 specimens (63.9-81.7 mm SL), same data as holotype.

Diagnosis

Melanotaenia sikuensis is distinguished from all of its congeners in the Birds Head region by: dorsal rays IV to V + I,14 to 15; anal rays I, 20 to 23; lateral scales 34 to 36; transverse scales 10-11; predorsal scales 15 to 18; cheek scales 12 to 17; gillrakers 13-15; head length, 24.7-26.1% of SL; a moderately short snout length, 8.1-8.9% of SL; interorbital width, 8.2-8.7% of SL; eye diameter, 7.1-8.0% of SL; prepelvic length, 36.7-39.0% of SL; a short predorsal length, 47.7-49.5% of SL; a short caudal peduncle length, 12.9-14.3; a moderately slender caudal peduncle depth, 10.1-11.5% of SL; a moderately short pelvic fin length, 15.5-19.3% of SL; a long dorsal fin base length, 38.5-42.1% of SL; a long second dorsal fin base length, 24.7-28.4% of SL; an intermediate anal fin base length 39.5-43.1, with a tall anal fin height 14.4-15.7% of SL; no stripe on the margin of the anal and dorsal fins; a continuous blue midlateral stripe, covering 2-3 scales wide from pectoral to middle sections and 3-4 scales rows on caudal section; dorsal origin behind anal origin, between 2nd to 3rd anal soft ray.

Description

Counts and proportions that appear in parentheses refer to the range for paratypes (based on 10 specimens, 63.9-81.7 mm SL) if different from the holotype. Morphometric data are given in table XIV.

Dorsal rays V + I,14 (IV-V + I,14-15); anal rays I,22 (I,20-23); pectoral rays 14 (13-15); pelvic rays I,5; lateral scales 34 (34-36); transverse scales 10 (10-11); predorsal scales 15 (16-18); cheek scales 14 (12-17); total gillrakers on first arch 15 (13-15); jaws about equal, oblique, premaxilla with an abrupt bend between the anterior horizontal portion and lateral part; maxilla ends below anterior edge of eye; lips thin; teeth conical with slightly curved tips extending to outer surface of lips; teeth of upper jaws in about 5-7 irregular rows, reduced to 1-2 rows posteriorly; lower jaws in about 6-7 irregular rows anteriorly, reduced to 1-2 rows posteriorly; several small conical teeth on vomer and palatines.

Scale of body cycloid, relatively large, and arranged in regular horizontal rows; scale margin slightly crenulate; predorsal scales extending to posterior portion of interorbital; preopercle scales from posterior angle to edge of eye arranged in 3 rows.

First dorsal fin origin behind anal fin origin, between 2nd to 3rd anal soft rays; tip of first dorsal fin reaching 2nd to 3rd soft ray of second dorsal fin; tip of second dorsal fin reaching 2/3 length of caudal peduncle for the males and half length for the

females; pelvic fin tips when depressed reaching base of 1st to 3rd soft anal fin ray for females and 3rd to 4th for males; caudal fin forked.

Colour of freshly collected male specimens: overall yellow; dorsal part of head and body yellow, belly yellowish; a continuous blue violet midlateral stripe of 2-3 scale wide from pectoral to middle sections and 3-4 scale rows on caudal section; pectoral fin translucent; remaining fins yellow dark; no margin stripe on dorsal and anal fins; a faint blue blotch extending from pectoral fin base to middle of anal fin; colour of females with equal intensity.

Sexual dimorphism: males have longer dorsal and pelvic fins but are not deeper bodied than females.

Comparisons

Melanotaenia sikuensis is part of the phylogenetic group "Southern Birds Head" species group and belongs to the "Birds Neck" species cluster. The new species is genetically allied to *M. angfa*, *M. parva*, *M. naramasae* sp. nov. and *M. rumberponensis* sp. nov. (Fig. 2). It is distinguishable from all of these species on the basis of the polymorphism of the 12 analyzed microsatellite loci (Fig. 8).

Melanotaenia sikuensis is distinguished from *M. angfa* and *M. parva* by the position of the dorsal fin origin in relation to the anal fin origin (dorsal behind anal origin *vs* dorsal before anal origin), by a shorter caudal peduncle length (12.9-14.3 *vs* 14.6-19.4% SL), and by the thickness of the midlateral stripe on the caudal peduncle (3-4 scales wide *vs* faint or absent). The new species differs from *M. angfa* by a shorter snout length (8.1-8.9 *vs* 9.0-9.5% SL) and by fewer gillrakers (13-15 *vs* 16-20). The new species differs from *M. parva* by a shorter predorsal length (47.0-49.5 *vs* 50.8-52.8% SL), a longer dorsal fin base length (38.5-42.1 *vs* 33.8-37.4% SL), a longer second dorsal fin base length (24.7-28.4 *vs* 20.7-24.7% SL), a longer anal fin base length (39.5-43.1 *vs* 33.1-38.2% SL) and by more dorsal soft rays (14-15 *vs* 11-13).

Melanotaenia sikuensis is distinguished from *M. naramasae* sp. nov. by a slender caudal peduncle depth (10.1-11.5 *vs* 11.8-12.4% SL), a shorter pelvic fin length (15.5-19.3 *vs* 19.4-23.5% SL) and by the dorsal origin which is vertically aligned between the 2nd and the 3rd anal soft ray for the former and between the 1st and the 2nd for the latter.

Melanotaenia sikuensis differs from *M. rumberponensis* sp. nov. by a shorter head length (24.7-26.1 *vs* 26.2-27.4% SL), a slender interorbital width (8.2-8.7 *vs* 9.1-9.5% SL), a smaller eye diameter (7.1-8.0 *vs* 8.7-9.3% SL), a shorter prepelvic length (36.7-39.0 *vs* 39.0-40.4% SL), a deeper anal fin height (14.4-15.7 *vs* 11.7-14.0% SL), and fewer gillrakers (13-15 *vs* 18).

Distribution and habitat

The new species is currently known only from the type locality, a small creek 5 m in width, and flowing eastward to Cendrawasih Bay. The habitat consists of a typical aquatic system in karstic environment.

Etymology

The species is named *sikuensis*, in reference to the river name at the type locality.

DISCUSSION

The mitochondrial phylogenetic tree combined with the microsatellite genotyping reveals previously undetected diversity among West Papuan rainbowfishes. Prior to this work, 24 valid species had been described from the region. Seven of the newly discovered species are confined to a single river system localized in fragmented karstic environments such as in the limestone karst formations from Ayamaru Plateau or in the northern extension of Lengguru Range. *Melanotaenia manibuii*, which was discovered on the southern foothills of the Arfak Mountains, is the only species distributed outside of a karstic formation.

Due to the intense tectonic movements of uplift or subsidence and the important erosion activity that happened during more than 10 million years, karst systems are characterized by endorheic drainages consisting of isolated lakes or fragmented rivers with aerial course which length varies from only several hundred metres to a few kilometres. These unique geological formations have therefore promoted an intense diversification and numerous speciation events in the Rainbowfishes from Western Papua (Kadarusman *et al.*, 2010, 2012b; Allen and Hadiaty, 2013; Allen *et al.*, 2014a, b). The total area covered during our field expeditions since 2007 represents approximately 20,000 km^2, which corresponds to less than 10% of the global terrestrial surface of Western Papua and to less than 20% of karst landscapes. Therefore, there are still many unexplored areas that remain to be studied, with a particular mention to the Central Birds Head lowlands eastward of Ayamaru Plateau between southern Tambrauw and Arfak Mountains and along the Northern foothills of Kumawa on Bomberai Peninsula.

The Lengguru fold-and-thrust Belt (LFTB) combined with the Central Dividing Range proved to have played a key role in the diversification of Melanotaeniidae at the regional scale (Kadarusman *et al.*, 2012a; Unmack *et al.*, 2013). Rapid tectonic uplift and subsidence raised in the Mid-Miocene the radiation of four distinct monophyletic clades, each restricted to a specific biogeographic province. The fact that all the species from Western Papua belonged to the same phylogenetic clade demonstrates their genetic relatedness and proves that dispersal with adjacent biogeographic provinces (*i.e.* Northern or Southern New Guinea) was disrupted since the geological formation of the LFTB. All the species

of melanotaeniids from Western New Guinea are endemic to the area. Because they are all obligate freshwater fish, further investigations on the diversity of other obligate freshwater taxa of this region appear very promising.

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Appendix. – Morphometric data for 20 *Melanotaenia* species from West Papua.

Refer to Allen and Hadiaty (2013) and Allen *et al.* (2014a, b) for morphometric data corresponding to *M. sneideri*, *M. flavipinnis*, *M. ericrobertsi* and *M. laticlavia*.

	Melanotaenia ajamaruensis					*Melanotaenia ammeri*					*Melanotaenia angfa*					*Melanotaenia arfakensis*				
	Material examined					Material examined					Material examined					Material examined				
SL (mm)	55.5-96.5					55.9-101.8					59.5-86.2					60.7-76.9				
in % standard length	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
Head length	30	23.1	25.1	24.4	0.6	14	24.6	27.8	25.8	0.9	13	25.2	26.5	25.7	0.4	10	23.9	25.3	24.6	0.5
Snout length	30	7.2	8.1	7.8	0.3	14	8.5	9.9	9.1	0.4	13	9.0	9.5	9.3	0.2	10	7.6	8.2	7.9	0.2
Interorbital width	30	7.4	8.5	8.1	0.3	14	8.2	8.8	8.5	0.2	13	8.6	9.4	8.9	0.2	10	8.8	9.3	9.0	0.2
Eye diameter	30	7.2	8.2	7.8	0.3	13	7.3	7.8	7.6	0.2	13	7.3	8.1	7.9	0.3	10	7.4	8.2	7.9	0.2
Body depth	30	29.6	40.0	35.3	2.7	14	29.7	38.7	33.3	2.8	13	28.8	32.3	31.0	1.0	10	30.1	36.6	33.0	1.9
Body width	30	12.9	18.6	14.9	1.7	14	11.7	14.2	12.7	0.7	13	13.6	16.0	14.6	0.7	10	13.0	14.6	13.7	0.5
Caudal peduncle depth	30	9.7	11.5	10.6	0.5	14	11.6	13.8	12.3	0.7	13	10.2	11.4	10.8	0.4	10	12.0	13.2	12.7	0.4
Caudal peduncle length	30	14.6	16.3	15.6	0.5	13	13.9	16.1	15.0	0.8	13	14.6	16.4	15.6	0.7	10	16.5	18.3	17.4	0.6
Predorsal length	30	45.3	50.0	47.9	1.1	14	49.8	52.5	50.9	0.7	13	45.9	49.5	48.1	1.1	10	47.1	49.6	48.3	0.8
Prepelvic length	30	36.3	39.5	38.1	0.9	13	38.5	40.5	39.3	0.6	13	36.8	40.0	38.7	1.0	10	36.4	39.1	37.9	0.9
Preanal length	30	49.4	53.9	51.9	1.4	14	49.7	53.3	51.0	0.9	13	49.4	52.1	50.6	1.0	10	47.7	50.1	48.7	0.8
Pectoral fin length	17	17.1	19.9	18.4	0.8	14	18.9	21.1	19.9	0.7	13	16.2	18.5	17.7	0.7	0				
Pelvic fin length	23	14.6	18.2	16.1	1.0	14	17.8	23.4	21.0	1.6	13	16.9	19.9	18.1	0.9	10	15.0	17.5	15.9	0.8
Spine length of first dorsal fin	24	8.6	11.6	10.3	0.9	14	8.7	12.0	10.3	0.9	13	10.3	11.7	10.9	0.4	8	9.4	12.1	10.9	1.0
Spine length of second dorsal fin	23	7.0	11.4	8.9	1.0	14	7.8	13.0	9.9	1.4	13	8.4	11.2	9.6	0.9	10	8.3	12.4	9.9	1.3
Spine length of anal fin	24	6.6	9.6	7.9	0.8	11	8.6	10.2	9.3	0.5	12	6.5	8.4	7.7	0.6	9	7.1	8.8	7.8	0.6
Dorsal fin base length	30	38.6	43.5	40.7	1.5	14	34.7	42.2	39.1	2.0	13	38.5	41.0	40.0	0.8	10	37.0	39.9	38.3	1.0
Second dorsal fin base length	30	24.1	29.3	26.4	1.2	14	22.1	28.6	25.2	1.9	13	24.1	28.6	26.2	1.1	10	24.2	27.0	25.5	0.9
Anal fin base length	30	33.9	40.4	37.7	2.0	14	37.4	44.1	40.4	2.0	13	35.4	40.0	38.2	1.3	10	39.7	43.6	41.3	1.3
Anal fin height						4	19.7	22.2	20.6	1.1										

Lateral scales	35-37	34-37	35-36	34-36
Transverse scales	7-8	9-10	10	10
Predorsal scales	15-16	14-19	16-18	14-18
Cheek scales	11-18	12-16	14-19	13-15
Dorsal rays	IV-VI + I,12-19	IV-VI + I,12-15	IV-V + I,14-16	IV-V + I,13-16
Anal rays	I,21-24	I,20-24	I,22-24	I,21-25
Pectoral rays	13-15	12-14	13-15	14-15
Pelvic rays	I,5	I,5	I,5	I,5
Total gillrakers on first arch	16-19	16-17	16-20	15-16

	Melanotaenia arguni					*Melanotaenia batanta*					*Melanotaenia boesemani*					*Melanotaenia catherinae*				
	Material examined					Material examined					Material examined					Material examined				
SL (mm)	54.8-72.6					73.9-110.0					54.7-86.9					58.1-79.8				
in % standard length	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
Head length	15	24.2	26.9	25.2	0.7	15	23.6	25.3	24.7	0.5	13	25.5	28.5	27.1	0.9	8	25.6	26.7	26.0	0.4
Snout length	15	8.3	9.1	8.7	0.3	15	9.2	9.5	9.4	0.1	13	7.8	8.7	8.4	0.3	8	8.5	9.1	8.8	0.2
Interorbital width	15	8.3	9.0	8.7	0.2	15	8.8	9.6	9.2	0.2	13	8.3	9.5	8.8	0.3	8	9.7	10.4	10.0	0.3
Eye diameter	15	6.6	7.2	7.0	0.2	15	7.0	7.8	7.5	0.2	13	8.6	10.7	9.5	0.5	8	8.5	9.1	8.9	0.2
Body depth	15	30.7	33.8	32.2	1.0	15	31.3	36.7	33.9	1.7	13	34.0	44.7	37.5	3.5	8	29.0	36.0	32.7	2.3
Body width	15	11.0	13.0	11.9	0.6	15	12.7	14.0	13.2	0.4	13	11.4	15.5	14.0	1.1	8	13.7	15.2	14.5	0.6
Caudal peduncle depth	15	10.0	10.8	10.4	0.3	15	11.6	12.9	12.2	0.4	13	9.8	12.4	10.9	0.9	7	13.5	14.9	13.9	0.5
Caudal peduncle length	15	14.1	16.1	15.4	0.6	14	14.1	15.5	14.9	0.4	13	15.4	18.7	17.1	1.0	8	14.3	15.5	15.0	0.4
Predorsal length	15	49.8	52.1	50.7	0.9	15	50.8	52.9	51.7	0.7	13	49.1	54.4	51.8	1.4	8	49.6	51.0	50.2	0.5
Prepelvic length	15	36.8	39.7	38.3	0.9	15	34.8	37.2	36.3	0.6	13	40.0	42.0	40.9	0.7	8	38.6	40.0	39.2	0.6
Preanal length	14	49.7	52.9	51.3	1.0	15	44.6	48.0	46.5	1.1	13	51.3	57.6	53.4	1.6	8	49.2	50.0	49.5	0.3
Pectoral fin length	15	18.1	20.9	19.5	0.7	14	19.1	21.1	20.3	0.7	3	16.9	18.8	17.7	1.0	0				
Pelvic fin length	15	16.2	20.2	17.7	1.2	15	21.1	25.2	22.5	1.2	13	13.8	16.7	14.8	1.0	2	17.6	17.9	17.8	0.2
Spine length of first dorsal fin	15	9.4	11.8	10.7	0.7	13	9.9	11.8	11.0	0.7	10	9.8	12.2	11.0	0.9	7	9.2	10.5	9.7	0.4
Spine length of second dorsal fin	15	8.6	12.1	10.4	1.2	14	8.1	11.3	9.6	0.9	10	7.5	11.0	8.7	1.0	7	8.7	9.4	9.1	0.3
Spine length of anal fin	15	7.2	9.5	8.5	0.6	12	7.1	9.9	8.8	0.9	9	7.4	9.7	8.4	0.9	3	7.5	8.6	8.1	0.5
Dorsal fin base length	14	34.7	41.4	38.0	2.1	15	36.3	39.2	37.1	0.9	12	33.5	37.4	36.1	1.1	8	38.0	39.7	38.7	0.6
Second dorsal fin base length	14	22.0	26.8	24.2	1.7	15	22.6	26.3	23.9	1.0	12	22.1	24.7	23.3	0.7	8	25.2	27.8	26.5	0.9
Anal fin base length	14	37.0	42.3	39.7	1.6	15	44.0	50.5	46.7	2.0	12	32.2	37.3	35.6	1.6	8	41.0	45.1	42.8	1.6
Anal fin height	15	11.3	16.3	13.5	1.3															

Lateral scales	34-37	34-35	33-36	32-33
Transverse scales	10-11	10	7-8	10
Predorsal scales	16-18	16-18	15-16	15-16
Cheek scales	14-20	11-15	10-12	13-17
Dorsal rays	IV-V + I,12-16	IV-VI + I,12-15	IV-V + I,11-14	IV-V + I,12-15
Anal rays	I,20-24	I,26-29	I,18-22	I,21-23
Pectoral rays	12-15	13-15	13-16	13-14
Pelvic rays	I,5	I,5	I,5	I,5
Total gillrakers on first arch	17-18	17-18	16-17	14-18

	Melanotaenia fasinensis					*Melanotaenia fredericki*					*Melanotaenia irianjaya*					*Melanotaenia kokasensis*				
	Material examined					Material examined					Material examined					Material examined				
SL (mm)	69.9-120.2					55.7-81.5					39.6-102.6					49.1-57.2				
in % standard length	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
Head length	13	23.1	25.0	24.5	0.5	11	23.7	25.4	24.5	0.5	14	25.3	27.0	25.9	0.5	3	25.9	26.7	26.2	0.4
Snout length	13	9.2	9.5	9.3	0.1	11	7.7	8.4	8.2	0.2	14	9.2	10.3	9.6	0.4	3	8.3	8.6	8.5	0.2
Interorbital width	13	7.6	8.2	7.9	0.2	12	8.0	8.4	8.2	0.1	14	8.3	9.4	8.9	0.3	3	9.0	9.2	9.1	0.1
Eye diameter	13	6.4	8.1	7.4	0.5	12	7.4	8.5	7.8	0.3	14	7.4	8.4	7.9	0.3	3	8.2	8.7	8.5	0.3
Body depth	13	27.8	37.9	33.0	2.5	11	26.3	33.0	29.8	2.2	13	28.6	38.1	33.6	2.7	3	30.1	31.4	30.8	0.6
Body width	13	12.1	14.2	12.8	0.5	11	11.5	12.9	12.4	0.4	12	12.5	15.3	13.5	0.9	3	11.3	11.8	11.6	0.3
Caudal peduncle depth	13	10.1	10.8	10.4	0.2	12	9.3	11.0	10.2	0.6	14	9.7	11.1	10.5	0.4	3	11.2	11.5	11.4	0.1
Caudal peduncle length	13	13.3	15.6	14.6	0.8	11	13.0	17.7	15.5	1.3	14	14.4	17.6	15.9	1.0	3	17.5	18.9	18.0	0.8
Predorsal length	13	48.1	49.8	48.8	0.6	12	47.7	51.9	50.1	1.3	13	50.0	52.5	51.2	0.7	3	49.6	49.7	49.7	0.1
Prepelvic length	13	34.5	38.8	36.2	1.1	12	33.9	39.5	36.5	1.9	11	36.8	38.7	37.6	0.6	3	37.8	38.5	38.1	0.4
Preanal length	13	45.6	48.4	46.8	0.9	12	45.4	53.0	48.1	2.5	13	48.5	52.5	50.1	1.2	3	50.2	50.6	50.4	0.2
Pectoral fin length	11	17.4	19.9	18.6	0.8	10	17.6	21.2	19.2	1.0	13	18.6	20.9	19.9	0.7	3	18.2	20.0	19.3	0.9
Pelvic fin length	11	17.0	19.5	18.5	0.8	11	16.1	19.3	17.1	0.9	9	20.6	24.9	22.4	1.5	3	16.3	17.9	17.0	0.8
Spine length of first dorsal fin	13	10.0	12.7	11.1	0.9	10	12.0	13.9	12.8	0.6	6	12.3	13.6	13.0	0.5	3	12.3	12.9	12.6	0.3
Spine length of second dorsal fin	13	7.3	11.6	9.3	1.2	9	11.7	13.7	12.6	0.7	6	12.3	14.1	13.2	0.7	3	10.8	13.1	11.9	1.2
Spine length of anal fin	12	7.0	8.8	8.1	0.7	9	8.9	9.6	9.2	0.2	6	9.8	11.6	10.5	0.6	3	8.7	9.8	9.1	0.6
Dorsal fin base length	13	39.0	42.2	40.4	1.1	11	34.7	38.8	36.8	1.4	12	35.0	37.7	36.5	1.0	3	33.6	37.1	35.5	1.8
Second dorsal fin base length	13	25.9	29.5	27.2	1.0	11	21.0	24.6	23.0	1.1	9	21.1	23.7	22.8	0.9	3	21.0	23.1	22.2	1.1
Anal fin base length	13	40.6	48.4	42.9	2.3	11	36.1	43.0	39.9	2.2	14	36.4	43.5	39.5	2.4	3	37.4	37.9	37.6	0.3
Anal fin height																				

Lateral scales	37-39	36-39	35-39	36-38
Transverse scales	9	7-8	10	10
Predorsal scales	17-19	16-19	17-19	16-17
Cheek scales	14-18	13-16	13-16	14
Dorsal rays	IV-VI + I,14-17	V-VI + I,12-16	IV-VI + I,12-16	IV-V + I,12-14
Anal rays	I,24-27	I,24-25	I,22-26	I,23
Pectoral rays	13-15	13-15	13-15	13-14
Pelvic rays	I,5	I,5	I,5	I,5
Total gillrakers on first arch	17-19	13-16	19-21	16-19

	Melanotaenia misoolensis					*Melanotaenia multiradiata*					*Melanotaenia parva*					*Melanotaenia salawati*				
	Material examined					Material examined					Material examined					Material examined				
SL (mm)	46.1-77.4					64.1-89.9					50.7-67.0					68.0-91.6				
in % standard length	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
Head length	8	25.4	26.6	26.1	0.5	14	24.5	26.3	25.5	0.5	8	25.3	27.6	26.3	0.8	16	23.9	25.1	24.7	0.4
Snout length	8	8.6	9.0	8.8	0.1	14	8.9	9.7	9.3	0.2	8	8.2	9.1	8.7	0.3	16	8.5	9.1	8.8	0.2
Interorbital width	8	9.0	9.5	9.3	0.2	14	7.7	8.3	8.0	0.2	8	8.7	9.5	9.1	0.2	16	8.6	9.2	8.9	0.2
Eye diameter	8	8.4	9.9	9.1	0.5	14	6.7	7.4	7.1	0.2	8	7.9	8.4	8.2	0.2	16	6.9	7.9	7.5	0.3
Body depth	8	29.1	34.1	31.7	1.7	14	27.1	30.5	28.7	1.0	7	32.7	37.5	35.0	1.8	16	32.1	37.9	34.4	1.6
Body width	8	12.5	14.1	13.1	0.5	14	11.2	12.6	12.0	0.4	8	13.8	15.9	15.1	0.7	16	12.5	13.7	13.3	0.3
Caudal peduncle depth	7	10.7	11.3	10.9	0.2	14	9.3	10.0	9.7	0.2	8	11.6	12.5	12.1	0.4	16	11.0	12.6	11.9	0.5
Caudal peduncle length	7	15.6	16.5	16.1	0.4	14	14.1	15.9	15.0	0.5	8	16.8	19.4	17.8	0.9	16	15.7	16.7	16.2	0.3
Predorsal length	8	48.1	51.2	49.5	0.9	13	49.5	50.9	50.3	0.4	7	50.8	52.8	51.3	0.7	16	51.4	54.8	52.2	0.9
Prepelvic length	7	37.9	39.1	38.5	0.4	14	36.3	38.6	37.5	0.7	8	38.3	41.0	39.8	1.1	16	34.5	37.2	35.9	0.6
Preanal length	8	48.5	51.4	49.4	0.9	14	48.6	50.8	49.8	0.7	8	50.6	53.3	52.1	0.9	16	44.2	48.1	46.3	1.1
Pectoral fin length	8	18.4	20.4	19.5	0.8	14	16.9	19.2	18.5	0.6	8	18.1	19.3	18.8	0.5	13	20.2	22.5	21.4	0.7
Pelvic fin length	8	16.9	19.7	18.0	1.1	14	16.4	21.8	17.9	1.5	8	15.1	17.3	16.1	0.8	15	18.5	22.0	20.4	1.1
Spine length of first dorsal fin	8	10.2	12.2	11.2	0.7	14	10.5	12.4	11.5	0.6	8	9.4	11.4	10.1	0.6	14	9.5	12.2	10.7	0.7
Spine length of second dorsal fin	8	9.4	13.3	10.5	1.2	14	10.0	13.6	11.4	1.0	8	8.1	10.7	8.9	0.9	15	8.6	11.7	10.2	1.0
Spine length of anal fin	8	9.0	11.1	9.7	0.7	13	7.9	10.0	8.9	0.6	7	7.1	8.9	7.7	0.7	15	7.4	9.7	8.7	0.7
Dorsal fin base length	8	37.3	40.5	38.5	1.1	14	34.7	38.7	36.4	1.0	8	33.8	37.4	36.1	1.2	16	33.7	36.1	35.1	0.7
Second dorsal fin base length	8	22.7	24.3	23.5	0.6	14	21.7	24.9	23.5	0.9	8	20.7	24.7	22.8	1.5	16	19.7	22.3	21.0	0.9
Anal fin base length	8	38.4	42.1	39.9	1.3	14	37.6	41.1	39.0	1.1	8	33.1	38.2	35.9	1.7	16	43.9	48.3	45.6	1.3
Anal fin height	4	13.7	15.5	14.6	0.7															

Lateral scales	34-36	36-39	34-36	36-37
Transverse scales	10	10-11	10	10-11
Predorsal scales	14-18	16-19	14-18	17-20
Cheek scales	13-16	13-17	10-13	16-20
Dorsal rays	IV-VI + I,13-14	IV-VI + I,13-16	V-VI + I,11-13	V-VI + I,12-14
Anal rays	I,23-26	I,24-27	I,19-23	I,25-28
Pectoral rays	13-14	15-16	12-14	14-16
Pelvic rays	I,5	I,5	I,5	I,5
Total gillrakers on first arch	16-19	18-19	13-16	17-18

	Melanotaenia synergos					*Melanotaenia urisa*					*Melanotaenia veoliae*					*Melanotaenia wanoma*				
	Material examined					Material examined					Material examined					Material examined				
SL (mm)	57.7-67.1					57.4-87.8					53.9-92.3					59.6-72.8				
in % standard length	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD	n	min	max	mean	SD
Head length	5	25.4	26.3	25.8	0.4	17	23.7	25.2	24.5	0.4	16	25.3	27.0	25.9	0.5	18	24.7	26.4	25.6	0.6
Snout length	5	9.2	9.6	9.3	0.2	17	7.6	8.7	8.4	0.3	16	8.0	9.1	8.7	0.3	18	8.0	9.1	8.6	0.3
Interorbital width	5	9.8	10.3	10.0	0.2	17	7.9	8.8	8.5	0.2	16	9.0	9.5	9.2	0.1	18	8.4	9.0	8.7	0.2
Eye diameter	5	7.9	8.3	8.1	0.1	17	8.0	9.0	8.4	0.3	16	6.3	7.9	7.4	0.4	18	7.3	7.8	7.6	0.1
Body depth	5	33.2	34.6	33.9	0.5	17	27.0	34.2	30.6	2.1	16	30.8	37.5	33.8	1.8	18	28.7	32.8	30.7	1.1
Body width	5	13.2	13.8	13.5	0.2	17	10.0	11.2	10.6	0.4	16	11.3	12.6	11.9	0.4	18	11.4	13.5	12.4	0.7
Caudal peduncle depth	5	13.5	14.1	13.8	0.2	17	8.6	10.0	9.6	0.4	16	10.3	11.7	11.0	0.5	18	10.0	11.1	10.6	0.3
Caudal peduncle length	5	15.8	18.2	17.2	1.0	17	16.3	19.0	17.4	1.0	16	16.5	17.7	16.9	0.4	18	14.0	16.1	15.4	0.6
Predorsal length	5	47.5	49.5	48.7	0.8	17	50.6	54.5	52.3	0.9	16	49.5	52.1	50.6	0.9	16	47.3	49.5	48.4	0.8
Prepelvic length	5	38.7	40.4	39.5	0.7	16	36.3	39.2	37.3	0.7	16	36.9	38.0	37.5	0.4	17	38.3	40.4	39.2	0.7
Preanal length	5	48.5	49.8	49.1	0.5	17	47.0	49.1	48.3	0.7	16	47.4	49.3	48.3	0.6	17	49.6	52.8	50.9	1.1
Pectoral fin length	0					15	15.2	17.4	16.2	0.6	13	18.1	20.7	19.5	0.7	18	18.7	20.7	19.6	0.6
Pelvic fin length	4	17.1	18.5	17.8	0.6	17	11.0	13.7	12.5	0.6	15	16.4	20.1	19.0	1.0	18	16.3	19.5	17.6	0.9
Spine length of first dorsal fin	4	8.3	8.9	8.7	0.3	17	9.0	12.8	10.7	1.2	15	10.2	12.8	11.7	0.7	17	10.2	12.1	11.3	0.5
Spine length of second dorsal fin	4	7.0	8.9	7.8	0.9	17	8.3	14.3	10.7	1.8	16	7.9	11.2	9.7	0.9	18	9.2	12.9	10.6	1.1
Spine length of anal fin	2	5.9	7.9	6.9	1.4	16	7.1	8.5	7.8	0.3	15	9.1	11.3	10.0	0.6	17	9.1	10.4	9.6	0.3
Dorsal fin base length	5	38.6	41.1	39.4	1.0	16	30.8	35.7	33.7	1.6	15	36.7	39.9	38.0	1.1	18	36.8	40.5	38.7	1.0
Second dorsal fin base length	5	25.2	27.3	26.1	0.8	16	20.4	23.5	22.0	1.0	15	23.2	25.8	24.3	0.9	18	24.3	27.5	25.6	1.1
Anal fin base length	5	41.6	43.3	42.3	0.7	16	35.4	41.3	38.1	1.6	15	37.9	45.1	41.3	1.8	18	36.0	41.4	39.3	1.3
Anal fin height						17	6.8	9.9	8.5	0.9	11	12.7	16.6	14.3	1.3	18	11.9	17.4	13.2	1.2

Lateral scales	32-33	36-39	34-37	34-36
Transverse scales	10	10-11	10-11	10-11
Predorsal scales	15-17	18-20	17-18	15-17
Cheek scales	13-18	13-16	12-14	14-18
Dorsal rays	IV-VI + I,11-12	IV-V + I,12-15	IV-V + I,12-13	IV-VI + I,14-15
Anal rays	I,18-21	I,21-23	I,22-27	I,22-25
Pectoral rays	13-14	13-14	12-13	13-14
Pelvic rays	I,5	I,5	I,5	I,5
Total gillrakers on first arch	14-18	17-18	18-20	15-16

ANNEXE 3

Research Article

Genetic diversity of Boeseman´s Rainbowfish (*Melanotaenia boesemani*) reared in Indonesian farms compared to endangered natural populations

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Abstract
Endemic to two lakes (Ayamaru and Uter) of West Papua (Indonesia), the Boeseman´s Rainbowfish *Melanotaenia boesemani* Allen & Cross, 1980 is a very popular ornamental freshwater fish. As a result, this rainbowfish species faces great threats and is on the red list of endangered species. Therefore, rearing of this species in aquaculture systems appears to be a promising solution to limit capture of wild specimens and prevent its extinction. Although its reproduction cycle has been controlled for more than 30 years, very few farms still raise *M. boesmani* , probably due to the problems reported by the farmers, *such as* decline of production, higher proportion of females per spawning, loss of coloration, lower growth rate and fecundity. Using 12 microsatellites previously developed for this species, comparison of genotypes within six farms around Jakarta indicated that all reared strains originated from Ayamaru Lake. No deficit in heterozygotes was evidenced, suggesting that there was no major inbreeding in these reared populations. Genotype analysis also suggested that *M. boesemani* species is a metapopulation composed of genetically differentiated populations. Altogether, these results indicate that the problems experienced by the farmers are due not to inbreeding depression but to other factors such as inadequate management and/or poor water quality. Yet, increasing aquaculture production is probably the most effective way to alleviate the pressure that *M. boesemani* faces in its natural environment.

Key-words: *Melanotaenia boesemani*, endangered species, aquaculture strains, genetic variability, microsatellites

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Introduction

The Boeseman´s Rainbowfish *Melanotaenia boesemani* Allen & Cross, 1980 is one of the most popular rainbowfish species in the aquarium hobby. When fully matured, males display a very distinct pattern of half-and-half coloration marked by a brilliant blue anterior and bright yellow to orange-red posterior (Fig. 1) [1, 2]. Gerald Allen discovered the species while studying the material collected in 1954-55 by Marinus Boeseman and stored at the National Museum of Natural History in Leiden (Netherlands) [3]. According to several studies [1, 4, 5], the species is only known from Ayamaru Lakes and tributaries and from Uter-Aitinyo Lake (or Uter L.). The two locations are 30 km apart and are separated by rugged karsts (Fig. 2). After the first publication describing *M. boesemani* [3], great interest arose in the potential commercial value of this species. It was introduced to the aquarium hobby in 1983 and has steadily increased in popularity since then [6]. In the mid-1980s, more than 60,000 males were caught and exported monthly from Ayamaru [7]. Such over-exploitation has therefore quickly brought this species to the verge of extinction in its natural habitat [4]. It has been on the red list of endangered species since 2004, and only aquaculture products are now supposed to be exported [8].

Although there is a lack of precise data, very few Indonesian farms, no more than ten, breed this species, all located around Jakarta, and their production does not account for the total exported fish. Boeseman´s Rainbowfish has been domesticated and produced in Indonesian farms since 1983. At present, farmers claim a decrease of both quantity and quality: males are not as colored as in the wild; growth rate and fecundity are slower; and morphological abnormalities frequently occur (unpublished data obtained from the farmers; Fig. 1c). They attribute these observations to loss of genetic variability and possible inbreeding (personal communication).

Twelve nuclear DNA microsatellite markers were recently developed in this species [9] for conservation purposes. We used these microsatellite markers to assess the genetic variability of six different strains of Boeseman's Rainbowfish reared in six Javanese farms. We compared their genetic variability with that of wild populations to determine the geographic origin of the founders and quantify possible loss of variability. Prior to these analyses, the microsatellite markers were further validated by testing their Mendelian inheritance through crossing experiments with five mate pairs of Boeseman's Rainbowfish obtained from a French retailer.

Methods

Fish sampling

A total of 432 *M. boesemani* specimens were analyzed in the present study. Wild fish were captured in 2007 by using gillnets at two distinct locations, respectively in the vicinity of Ayamaru lake (Tiwit tributary, 1°15.463'S and 132°14.939'E, 28 specimens) and at the Uter lake (1°25.957'S and 132°23.258'E, 49 specimens) (Fig. 2). Specimens were captured under local permits obtained by Akademi Perikanan Sorong (APSOR, West Papua). Captive fish were obtained from six farms located around Jakarta. These six farms have been cultivating *M. boesemani* for many years (up to 30 years), do not practice out-crossings with other breeders, and are facing difficulties such as more females in each harvest (only males are suitable for sale), smaller body size, and loss of color brightness. Approximately 30 individuals were collected from each farm. For Mendelian inheritance tests, five breeding pairs were selected from stock purchased from a French ornamental fish retailer (Botanic, Montpellier) and reared in distinct 60L aquariums. Progeny of each parental pair were collected within the two following months, and consisted of 23 to 43 hatchlings, depending on the spawn size. All fish were anaesthetized with 0.1 mL/L Eugenol (in accordance with the EU Directive 2010/63/EU) and a ~1-cm^2 piece of anal fin was collected and stored in absolute ethanol for further DNA extractions. Fish were then allowed to recover from anaesthetic and were released back into either the lakes (at the sampling site) or fish ponds. Experiments on captive animals were conducted at the aquatic experimental facilities of ISEM (PLATAX) (Montpellier) under the laboratory agreement for animal experimentation number A-34-172-24 and the author's personal authorization for animal experimentation number 34-188, both provided by the French government.

DNA extraction and microsatellite amplification

Individual samples were genotyped with 12 nuclear microsatellite markers that were recently developed [9]. DNA was extracted from a small fragment (10 mg) of anal fin clip with the NucleoSpin® 96 Tissue kit (Macherey-Nagel), according to the manufacturer's instructions, using a Janus automated Workstation (Perkin Elmer). Forward primers were end-labeled with fluorescent dyes (5'FAM, 5'HEX, 5'ATO550, 5'ATO565) (Eurofins). Each reaction contained 5 µl of 2x Master mix (Fast-Start PCR kit, Roche), 0.1 µM of forward primer, 0.4 µM of reverse primer, and 0.5 µl of template DNA. Cycling conditions were as follows: initial denaturation at 95°C for 4 min, followed by 30 cycles of 95°C for 20 s, 56°C for 20 s, and 72 °C for 30 s, and a final elongation step of 7 min at 72°C. Amplicon size was analyzed by capillary electrophoresis as previously described [9], in the technical facilities of the labex "Centre Méditerranéen de l'Environnement et de la Biodiversité" (Montpellier). Allele sizing and genotyping were achieved with the Peak Scanner v1.0 and GeneMapper® v5.0 software (Applied Biosystems).

Genetic diversity analysis

Allelic numbers (Na), average observed (Ho) and expected (He) heterozygosities were calculated using the GENETIX 4.05 software [10]. Data were analyzed for possible departures from Hardy-Weinberg equilibrium by estimating the significance of the multilocus inbreeding coefficient (Fis) with 1,000 random allelic permutations of the original datasets. The genetic structure among the investigated populations was also evaluated by calculating pairwise Fst values with 1,000 random permutations. The significance level of *P* value for Fis and Fst was defined as the probability of obtaining absolute values higher than or equal to the observed one under the null hypothesis. The genetic relationships between multilocus genotypes of the six reared strains and the wild populations of *M. boesemani* from Ayamaru and Uter Lakes were also assessed, using a factorial correspondence analysis (FCA) available in Genetix 4.05 software.

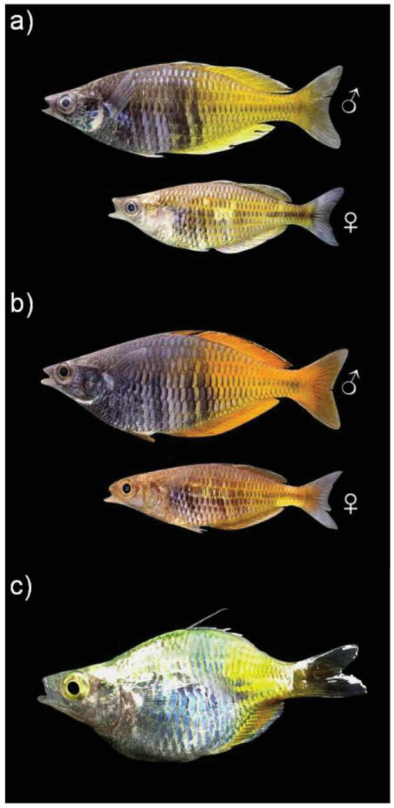

Fig. 1. Coloration patterns of Boeseman´s Rainbowfish *Melanotaenia boesemani.* Panels (a) and (b) show the difference between wild male and female specimens from Ayamaru and Uter Lakes (West Papua, Indonesia), respectively; panel (c) shows a reared specimen from Gusi farm (Jakarta) with a malformation, as frequently observed in the investigated farms.

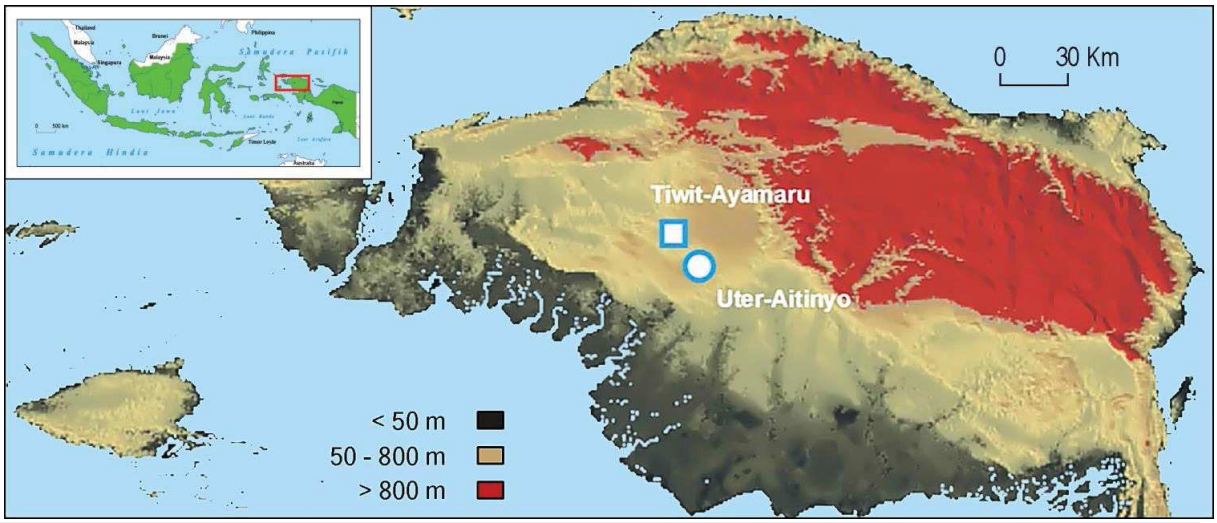

Fig. 2. Geographic localization of Ayamaru and Uter Lakes in West Papua, Indonesia.

Results

Experimental validation of the DNA microsatellite markers

The Mendelian inheritance of each microsatellite marker and the presence of putative null alleles were evaluated through crossing experiments. For this purpose, the genotypes of the progenies were resolved and compared to those of their corresponding parents. Results indicated that all genotypes observed in the offspring matched those expected from the parental ones: there were no heterozygous genotypes different from the predicted ones, genotype frequencies were similar to those expected (Appendix 1), and there was no significant difference between the observed and expected heterozygosities calculated on all loci for each crossing population (Table 1). Therefore, all 12 microsatellite loci seemed subject to Mendelian inheritance and there are no null alleles at these loci.

Table 1. Observed (Ho) and expected (He) heterozygosity calculated on all loci for each crossing population and associated Fis.

	Crossing 1	Crossing 2	Crossing 3	Crossing 4	Crossing 5
Ho ± SD	0.65 ± 0.27	0.69 ± 0.32	0.67 ± 0.30	0.56 + 0.26	0.70 ± 0.38
He ± SD	0.52 ± 0.19	0.49 ± 0.21	0.47 ± 0.19	0.43 ± 0.18	0.49 ± 0.25
Fis	NS	NS	NS	NS	NS

NS: non-significant

Genetic variability and heterozygosity of M. boesemani in aquaculture settings and in natural populations

A total of 151 alleles were detected in the 183 farmed and 77 wild specimens in the 12 microsatellite loci. The total number of alleles per locus for all populations varied from 5 to 22, and all loci were polymorphic in each strain and wild population (Appendix 2). Generally, the domesticated populations showed a lower genetic variability (total number of alleles between 62 and 86) compared to the Ayamaru wild population (107 alleles), but comparable to that observed in the wild population of Uter (82 alleles) (Table 2). Considering only the wild populations, there were 23 private alleles for the Uter Lake population and 48 private alleles for Ayamaru Lake. When all samples were included, there were 17 private alleles for Uter Lake, 16 private alleles for Ayamaru Lake and 21 private alleles for the domesticated populations (*i.e.*, only present in one or more strains and absent in wild populations). There were six alleles shared between Uter Lake and one or more strains, and 32 alleles shared between Ayamaru lake and at least one strain. There were 51 alleles shared between both wild populations and one or more strains, and eight alleles shared between Ayamaru and Uter lakes but absent from any strains (Appendix 2). Likewise, the heterozygosities calculated for the captive populations (Hobs comprised between 0.53 and 0.64) were lower than that calculated for the Ayamaru wild population (0.68) but similar to that of Uter population (0.56) (Table 2). Moreover, the Ho were never significantly different from the He. The multilocus Fis values for all populations (wild and captive) were between -0.010 and 0.061, and none of them was significant except that for the Sukri strain (Fis = 0.061, $P < 0.05$) (Table 2). These results strongly suggest that, with the exception of the Sukri farm, there is no deficit in heterozygotes in any population. Finally, the pairwise Fst values calculated between each population were all significant ($P < 0.05$), except between Warso and Yahya and between Warso and Didi farms, indicating that most of the populations are genetically differentiated from each other (Table 3).

Table 2. Sample size (N), allele number (Na), average observed (Ho) and expected (He) heterozygosity, and multilocus Fis values for each wild population and strain

Population	N	Na	Ho	He	Fis
Uter lake	49	82	0.56	0.55	-0.010
Ayamaru lake	28	107	0.68	0.69	0.013
Farm Gusi	34	79	0.61	0.61	-0.007
Farm Sukri	30	74	0.59	0.63	0.061*
Farm Hasan	30	86	0.64	0.64	-0.001
Farm Yahya	30	75	0.61	0.62	0.010
Farm Didi	29	62	0.53	0.55	0.029
Farm warso	30	71	0.62	0.61	-0.013

* $P < 0.05$

Table 3. Population differentiation based on pairwise Fst estimates

	Ayamaru Lake	Gusi	Sukri	Hasan	Yahya	Didi	Warso
Uter Lake	0.176***	0.228***	0.226***	0.221***	0.249***	0.263***	0.231***
Ayamaru Lake		0.030***	0.028***	0.028***	0.062***	0.075***	0.054***
Gusi			0.012***	0.032***	0.057***	0.055***	0.053***
Sukri				0.015**	0.029***	0.038***	0.032***
Hasan					0.030***	0.036***	0.028***
Yahya						0.011*	0.002 NS
Didi							0.007 NS

NS: non significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Geographic origin of M. boesemani reared in Indonesian farms

A factorial correspondence analysis (FCA) was performed on all multilocus genotypes obtained for the six captive and the two wild populations (Fig. 3). The projection made on axis 1 (5.99%) and axis 2 (2.47%) indicated that the wild population from Uter Lake could be genetically differentiated from that of Ayamaru Lake. Moreover, all the fish collected from the farms (*i.e*. polygon "pooled strains") were partly grouped with those of Ayamaru Lake (*i.e*., polygon "Ayamaru lake"). The third axis of the FCA did not enable us to separate the farm samples from the Ayamaru wild population. Likewise, when the Uter population was removed, the Ayamaru and farm specimens still overlapped. These results suggest that all captive fish originate from the region of Ayamaru Lake.

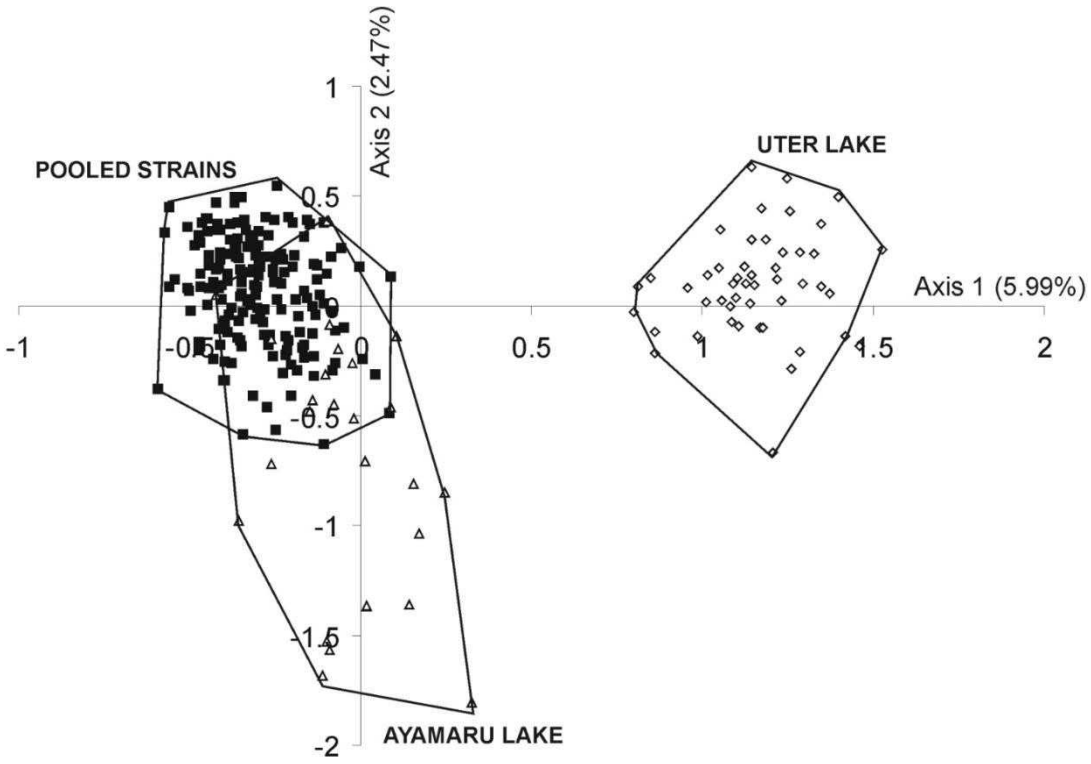

Fig. 3. Factorial Component Analysis based on the multilocus genotypes of Boeseman's Rainbowfish *Melanotaenia boesemani* individuals from the 2 wild populations of Ayamaru (n=28) and Uter (n=49) and the 6 Indonesian farms (n=29-34).

Discussion

The possible presence of null alleles in the 12 microsatellite markers had been checked *in silico* and results suggested that *M. boesemani* species was not affected [9]. However, this needed to be experimentally validated in order to definitely exclude this possibility. The cross-breeding experiments performed in the present study showed that the 12 microsatellite markers follow a Mendelian inheritance, and ruled out the existence of null alleles due to primer misamplifications, as well as genotyping errors due to stuttering or large allele dropout.

Ayamaru and Uter lakes are located in Indonesia in the western part of New Guinea Island. These two lakes are not connected, are 30 km apart, and are separated by rugged karsts. Though they are considered to be the same species, the fish from these two lakes display significant morphological differences: those from Ayamaru lake have a bright blue anterior and bright yellow anterior coloration, whereas those from Uter show a bright blue anterior and a reddish posterior coloration (Fig. 1). Nevertheless, previous analyses based on both the mitochondrial cytochrome oxydase I gene and morphomeristic traits failed to separate these two populations, which are slightly overlapping [11]. Here, the use of 12 microsatellite markers brought clear evidence that they are genetically differentiated (Fig. 3), and emphasizes that population mixtures should be strictly avoided during any upcoming broodstock constitution. From the samples investigated here, many private alleles were evidenced between the two wild populations of Boeseman's Rainbowfish (23 for Uter lake and 48 for Ayamaru lake). Among the 12 markers, Mb_di1, Mb_di3, Mb_di4 and Mb_tetra2 accounted for more than 50% of the private alleles found between the two populations (42 over 71).

Among the investigated farms, Gusi, Hasan and Didi started to exploit Boeseman's Rainbowfish in 1983 and 1986, respectively, soon after the description of *M. boesemani* species by Allen and Cross [3] and the publication of *Rainbowfishes of Australia and Papua New Guinea* [12]. This book greatly increased the popularity of rainbowfishes, especially the newly discovered New Guinea species, including Boeseman's Rainbowfish [2]. In spite of the establishment of these farms, exploitation of this species from its natural environment in Ayamaru has persisted until now. The geography of Uter Lake, surrounded by mountains and characterized by steep and rocky shores, is less favorable to fishing activities than that of Ayamaru, which is more easily accessible. This is probably why all investigated domesticated populations originated from Ayamaru.

The core population of Ayamaru sampled 30 years ago for the cultivated strains was genetically different from that of the wild population sampled in 2007. This was especially evidenced by the 21 private alleles observed in the strains and absent in the wild population of Ayamaru Lake. The presence of these private alleles may suggest a loss of variability that the wild population of Ayamaru has undergone over the last 30 years. However, the fact that heterozygosity and allele number values calculated from the Ayamaru population are comparable with those of other rainbowfish species [9] suggests little or no loss of genetic diversity. Most likely, these private alleles indicate that the genetic diversity of the Ayamaru specimens investigated here are not representative of the whole genetic diversity of Boeseman's Rainbowfish in this area (Ayamaru Lake and tributaries). The wild fish sampled in 2007 were indeed collected in Tiwit River, a tributary of Ayamaru Lake, and could represent a distinct genetic entity from the whole population. In this case, the species could be a metapopulation composed of several population subdivisions according to their geographic distribution in the mosaic of habitats characterizing Ayamaru Lake.

It was recently shown in a wide range of marine fishes that overfishing could result in significant reduction of genetic diversity [13]. Another recent meta-population study using many marine fish species demonstrated that the renewal of a fish population depends much less on the amount of available genitors than on environmental factors [14]. If the data presented here are not sufficient to tackle these questions, they nonetheless highlight the urgent need for a complete and exhaustive sampling campaign to determine the exact genetic structure and diversity of this endangered species, in order to ensure its conservation. This would require collection of fish in many more locations of Ayamaru Lake and its tributaries. Indeed, in addition to overfishing, Ayamaru Lake also experiences important environmental threats (deforestation, urbanization) and episodic droughts. Based on the sequence of a mitochondrial DNA fragment of the native Australian freshwater fish *Rhadinocentrus ornatus*, Mather *et al*. showed that habitat degradation caused by urbanization significantly reduced genetic diversity [15].

Finally, the genetic variability within the six farmed populations was comparable to that of the natural population of Uter Lake. Because no deficit in heterozygotes was evidenced, there was no major inbreeding in these reared populations. Therefore, the problems experienced by the farmers (*i.e.,* decline of production, higher proportion of females per spawning, loss of coloration, lower growth rate and fecundity, morphological abnormalities) are obviously not due to inbreeding depression and are probably caused by other factors such as poor management and/or poor water quality. It is noteworthy that all investigated farms are located in industrial and densely populated peri-urban areas. As Jakarta and its suburbs have no waste water treatment system, the water used for rearing these fish is probably of very poor quality, and may contain many chemical and hormonal pollutants, both of which have been proven to alter major fish traits such as reproduction and growth. Indeed, exposure to low concentrations of endocrine disruptive chemicals (such as estrogens) can impede gonadal function, reduce fertilization success, decrease fecundity, alter mating behavior, and reverse sex of various

aquatic species [16-18]. Such exposure can even cause the collapse of fish populations at trace concentrations [19].

Regarding the coloration pattern, rainbowfish are able to change color according to the turbidity level of their environment [20, 21]. For instance, increased brightness of red colors in environments rich in organic matter (*i.e.,* more turbid) may enhance conspicuousness, allowing individuals to maintain communication in altered visual environments. Thus, the alteration of fish color brightness observed in reared animals may be an adaptation to the aquaculture conditions, where ponds are very shallow and mimic habitats with full-spectrum lighting. However, a better understanding of the visual system of rainbowfish is required to predict how changes in the aquatic light environment affect the physiology and ecology of these fishes and allow farmers to adapt their rearing conditions to maintain bright body colors.

Implications for conservation

The combination of important levels of biodiversity with low human population density (2-6 inhabitants / km^2) led Conservation International in 1997 to declare New Guinea as the only "*Major Tropical Wilderness Area*" remaining in Asia. For illustration, all the species of melanotaeniids from Western New Guinea are endemic to the area. Because many rainbowfish species have restricted distributions and are confined to specific habitats, such as isolated lacustrine environments or small parts of a single river system, they are highly vulnerable to environmental disturbance and over-harvesting. Ayamaru Lake is affected by the development of residential areas and ecotourism. Forest clearance has increased channel obstructions by sediments and led to partial drying of the lake. Meanwhile, simultaneous over-catching of the endemic Boeseman's Rainbowfish drove this species to the verge of extinction [7]. The results presented here (*i.e.,* no significant loss of heterozygotes in the wild populations and no inbreeding depression in the reared strains) indicate that, in spite of the threats that Ayamaru is facing, it is still possible to prevent the extinction of Boeseman's Rainbowfish. This, however, would require increased aquaculture production in order to quickly alleviate the overfishing pressure. This, in turn, would require better management of the quality of waters used for rearing Boeseman's Rainbowfish, which is a general concern in Indonesia.

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Appendix 1. Allele frequencies for each breeding pair (crossing) and its corresponding offspring at the 12 microsatellite loci.

Allele	Crossing and corresponding offspring														
	M1	F1	O1 (n=24)	M2	F2	O2 (n=40)	M3	F3	O3 (n=23)	M4	F4	O4 (n=32)	M5	F5	O5 (n=43)
Mb_di1															
95	-	-	-	-	-	-	0.50	-	0.28	-	-	-	0.50	-	0.29
97	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.21
109	-	-	-	0.50	0.50	0.55	-	-	-	-	-	-	-	-	-
113	1.00	0.50	0.67	-	0.50	0.21	-	1.00	0.50	1.00	0.50	0.70	-	0.50	0.24
117	-	0.50	0.33	0.50	-	0.24	0.50	-	0.22	-	0.50	0.30	-	-	-
119	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.26
Mb_di2															
113	-	0.50	0.33	-	-	-	0.50	1.00	-	-	-	-	-	-	-
123	-	-	-	0.50	-	0.35	-	-	-	0.50	-	0.22	-	-	-
127	-	0.50	0.17	-	-	-	-	-	-	-	-	-	-	-	-
129	-	-	-	-	0.50	0.20	-	-	-	0.50	1.00	0.78	-	0.50	0.26
133	-	-	-	0.50	-	0.15	-	-	0.72	-	-	-	-	-	-
135	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.23
139	0.50	-	0.33	-	0.50	0.30	0.50	-	0.28	-	-	-	-	-	-
141	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.50	0.51
143	0.50	-	0.17	-	-	-	-	-	-	-	-	-	-	-	-
Mb_di3															
153	-	1.00	0.50	-	-	-	0.50	0.50	0.46	0.50	0.50	0.48	-	-	-
161	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.25
163	0.50	-	0.25	1.00	0.50	0.80	0.50	0.50	0.54	-	-	-	1.00	-	0.50
165	-	-	-	-	0.50	0.20	-	-	-	-	-	-	-	-	-
171	0.50	-	0.25	-	-	-	-	-	-	0.50	0.50	0.52	-	0.50	0.25
Mb_penta1															
160	-	-	-	-	-	-	0.50	1.00	0.65	-	-	-	0.50	-	0.29
180	-	-	-	-	-	-	-	-	-	0.50	0.50	0.59	-	-	-
185	-	-	-	-	1.00	0.50	0.50	-	0.35	-	-	-	-	0.50	0.24
190	0.50	0.50	0.56	-	-	-	-	-	-	0.50	-	0.19	-	-	-
195	0.50	-	0.23	-	-	-	-	-	-	-	-	-	-	-	-
200	-	0.50	0.21	1.00	-	0.50	-	-	-	-	0.50	0.22	0.50	-	0.21
205	-	-	-	-	-	-	-	-	-	-	-	-	-	0.50	0.26
Mb_di4															
112	0.50	-	0.19	0.50	-	0.26	1.00	0.50	0.76	-	0.50	0.28	-	1.00	0.50
114	0.50	0.50	0.52	-	0.50	0.23	-	0.50	0.24	1.00	0.50	0.72	-	-	-
124	-	-	-	0.50	0.50	0.51	-	-	-	-	-	-	-	-	-
140	-	0.50	0.29	-	-	-	-	-	-	-	-	-	-	-	-

164	-	-	-	-	-	-	-	-	-	-	-	-	1.00	-	0.50
Mb_tetra1															
143	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.74	-	1.00	0.50	0.50	0.50	0.47
147	-	-	-	-	-	-	0.50	-	0.26	0.50	-	0.23	0.50	0.50	0.53
157	-	-	-	-	-	-	-	-	-	0.50	-	0.27	-	-	-
Mb_tri1															
113	1.00	0.50	0.71	0.50	1.00	0.75	1.00	0.50	0.76	1.00	1.00	1.00	1.00	1.00	1.00
116	-	0.50	0.29	-	-	-	-	-	-	-	-	-	-	-	-
119	-	-	-	0.50	-	0.25	-	0.50	0.24	-	-	-	-	-	-
Mb_tri2															
138	0.50	-	0.27	-	1.00	0.50	-	-	-	0.50	0.50	0.39	-	1.00	0.50
144	-	-	-	0.50	-	0.22	-	1.00	0.50	0.50	0.50	0.61	1.00	-	0.50
147	0.50	1.00	0.73	-	-	-	0.50	-	0.43	-	-	-	-	-	-
150	-	-	-	0.50	-	0.28	0.50	-	0.07	-	-	-	-	-	-
Mb_di5															
83	-	-	-	-	-	-	0.50	-	0.28	0.50	-	0.30	0.50	0.50	0.53
89	-	-	-	0.50	-	0.26	-	-	-	-	-	-	0.50	-	0.26
91	0.50	-	0.19	-	0.50	0.24	-	0.50	0.24	-	0.50	0.27	-	0.50	0.21
95	-	-	-	-	-	-	-	0.50	0.26	-	0.50	0.23	-	-	-
97	-	0.50	0.27	0.50	-	0.24	-	-	-	0.50	-	0.20	-	-	-
105	0.50	0.50	0.54	-	0.50	0.26	0.50	-	0.22	-	-	-	-	-	-
Mb_tetra2															
180	-	-	-	-	0.50	0.29	-	-	-	-	-	-	0.50	-	0.28
188	-	-	-	-	-	-	-	0.50	0.20	-	-	-	-	-	-
192	-	-	-	-	-	-	-	-	-	-	-	-	0.50	-	0.22
204	-	0.50	0.25	-	0.50	0.21	1.00	-	0.50	0.50	1.00	0.77	-	1.00	0.50
208	0.50	-	0.31	-	-		-	-	-	0.50	-	0.23	-	-	-
212	0.50	0.50	0.44	1.00	-	0.50	-	-	-	-	-	-	-	-	-
256	-	-	-	-	-	-	-	0.50	0.30	-	-	-	-	-	-
Mb_tri3															
133	0.50	0.50	0.56	1.00	0.50	0.76	1.00	1.00	1.00	0.50	1.00	0.72	1.00	1.00	1.00
142	0.50	0.50	0.44	-	0.50	0.24	-	-	-	0.50	-	0.28	-	-	-
Mb_tri4															
99	0.50	0.50	0.35	1.00	0.50	0.73	0.50	0.50	0.50	0.50	-	0.23	0.50	-	0.116
102	0.50	-	0.35	-	0.50	0.28	-	0.50	0.28	0.50	1.00	0.77	-	0.50	0.186
111	-	0.50	0.29	-	-	-	0.50	-	0.22	-	-	-	0.50	0.50	0.698

M: male; F: female; O: offspring

Appendix 2. Number of alleles per locus for each population

Locus	Uter Lake n=49	Ayamaru Lake n=28	Gusi farm n=34	Sukri farm n=30	Hasan farm n=30	Yahya farm n=30	Didi farm n=29	Warso farm n=30	Total number of alleles
Mb_di1	9	14	8	8	10	9	9	8	19
Mb_di2	13	14	9	8	10	9	9	9	17
Mb_di3	10	8	7	5	7	7	5	6	18
Mb_penta1	6	8	8	7	7	7	6	6	10
Mb_di4	10	15	10	8	11	12	7	13	22
Mb_tetra1	6	7	3	4	6	5	2	3	10
Mb_tri1	4	4	4	3	3	2	2	2	6
Mb_tri2	3	4	4	4	4	5	3	4	6
Mb_di5	6	8	8	9	8	7	6	7	12
Mb_tetra2	10	18	10	11	12	6	6	7	21
Mb_tri3	3	3	3	3	4	2	3	2	5
Mb_tri4	2	4	5	4	4	4	4	4	5

Appendix 3. Allelic frequency for each population

Locus	Uter Lake	Ayamaru lake	Gusi farm	Sukri farm	Hasan farm	Yahya farm	Didi farm	Warso farm
Mb_di1								
87	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
89	0.020	0.036	0.000	0.000	0.000	0.000	0.000	0.000
95	0.357	0.125	0.206	0.183	0.117	0.067	0.017	0.050
97	0.061	0.018	0.044	0.083	0.017	0.000	0.000	0.000
101	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
103	0.000	0.000	0.147	0.050	0.100	0.033	0.000	0.017
105	0.000	0.036	0.000	0.000	0.017	0.000	0.052	0.000
107	0.000	0.089	0.000	0.000	0.000	0.000	0.000	0.000
109	0.378	0.018	0.088	0.067	0.067	0.150	0.103	0.233
111	0.031	0.000	0.000	0.000	0.000	0.033	0.017	0.050
113	0.071	0.250	0.235	0.450	0.433	0.500	0.448	0.367
115	0.010	0.036	0.029	0.000	0.000	0.050	0.069	0.000
117	0.061	0.304	0.221	0.117	0.100	0.050	0.121	0.083
119	0.000	0.018	0.029	0.033	0.117	0.100	0.138	0.133
121	0.000	0.000	0.000	0.017	0.017	0.017	0.000	0.067
125	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
127	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
137	0.000	0.000	0.000	0.000	0.017	0.000	0.035	0.000
139	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
Mb_di2								
113	0.000	0.089	0.029	0.033	0.167	0.033	0.121	0.033
117	0.083	0.000	0.029	0.083	0.067	0.000	0.017	0.000
119	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
123	0.000	0.054	0.059	0.000	0.050	0.000	0.017	0.017
125	0.031	0.018	0.000	0.000	0.000	0.000	0.000	0.000
127	0.021	0.036	0.044	0.083	0.017	0.000	0.000	0.000
129	0.260	0.036	0.029	0.150	0.067	0.083	0.103	0.067
131	0.042	0.054	0.000	0.000	0.017	0.100	0.000	0.133
133	0.010	0.071	0.147	0.100	0.067	0.083	0.052	0.033
135	0.031	0.268	0.279	0.250	0.200	0.300	0.172	0.217
137	0.333	0.071	0.000	0.000	0.167	0.167	0.121	0.217
139	0.115	0.161	0.279	0.267	0.183	0.167	0.345	0.250
141	0.021	0.054	0.103	0.033	0.000	0.033	0.052	0.033
143	0.021	0.036	0.000	0.000	0.000	0.000	0.000	0.000
145	0.000	0.036	0.000	0.000	0.000	0.033	0.000	0.000
147	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000
149	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mb-di3								
145	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000
153	0.160	0.107	0.061	0.167	0.133	0.100	0.086	0.117

155	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000
157	0.000	0.000	0.015	0.033	0.050	0.017	0.000	0.033
159	0.000	0.107	0.000	0.000	0.000	0.017	0.000	0.000
161	0.000	0.179	0.015	0.000	0.017	0.033	0.035	0.017
163	0.000	0.446	0.788	0.650	0.650	0.550	0.603	0.433
165	0.021	0.071	0.030	0.000	0.033	0.033	0.017	0.067
167	0.000	0.018	0.000	0.033	0.033	0.000	0.000	0.000
169	0.011	0.054	0.000	0.000	0.000	0.000	0.000	0.000
171	0.000	0.000	0.076	0.117	0.083	0.250	0.259	0.333
173	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
175	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000
179	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
181	0.521	0.000	0.000	0.000	0.000	0.000	0.000	0.000
183	0.032	0.000	0.000	0.000	0.000	0.000	0.000	0.000
185	0.096	0.000	0.000	0.000	0.000	0.000	0.000	0.000
187	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mb_Penta 1								
160	0.644	0.268	0.368	0.167	0.133	0.017	0.018	0.000
165	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
170	0.000	0.054	0.000	0.000	0.000	0.000	0.000	0.000
175	0.000	0.000	0.147	0.067	0.083	0.067	0.036	0.069
180	0.000	0.071	0.074	0.100	0.167	0.283	0.286	0.138
185	0.011	0.214	0.044	0.083	0.217	0.217	0.232	0.259
190	0.078	0.232	0.191	0.267	0.200	0.017	0.036	0.138
195	0.167	0.125	0.029	0.033	0.133	0.067	0.000	0.017
200	0.033	0.018	0.132	0.283	0.067	0.333	0.393	0.379
205	0.067	0.000	0.015	0.000	0.000	0.000	0.000	0.000
Mb_di4								
98	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000
100	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
106	0.020	0.000	0.029	0.000	0.000	0.000	0.000	0.000
110	0.000	0.000	0.074	0.000	0.000	0.000	0.000	0.017
112	0.296	0.268	0.324	0.483	0.150	0.433	0.328	0.283
114	0.378	0.143	0.147	0.083	0.283	0.117	0.103	0.150
116	0.051	0.036	0.000	0.000	0.000	0.017	0.000	0.017
118	0.031	0.089	0.044	0.033	0.017	0.017	0.000	0.117
122	0.010	0.000	0.000	0.000	0.017	0.017	0.000	0.000
124	0.143	0.143	0.132	0.217	0.283	0.200	0.276	0.217
126	0.031	0.036	0.015	0.017	0.000	0.050	0.172	0.083
128	0.031	0.054	0.000	0.000	0.017	0.000	0.000	0.000
130	0.000	0.054	0.000	0.000	0.000	0.000	0.000	0.000
132	0.000	0.018	0.000	0.000	0.017	0.067	0.069	0.033
134	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.017
138	0.000	0.018	0.000	0.017	0.000	0.000	0.000	0.000

140	0.000	0.000	0.162	0.033	0.033	0.017	0.000	0.017
142	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000
144	0.000	0.018	0.000	0.000	0.067	0.000	0.017	0.000
148	0.000	0.018	0.000	0.000	0.100	0.033	0.000	0.017
152	0.000	0.036	0.000	0.000	0.000	0.017	0.000	0.017
164	0.000	0.000	0.059	0.117	0.017	0.017	0.035	0.017
Mb_Tetra 1								
131	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
135	0.010	0.036	0.000	0.000	0.017	0.000	0.000	0.000
137	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000
139	0.184	0.036	0.000	0.000	0.000	0.000	0.000	0.000
143	0.735	0.607	0.779	0.717	0.741	0.767	0.931	0.833
147	0.031	0.125	0.177	0.217	0.052	0.150	0.069	0.117
149	0.000	0.071	0.044	0.000	0.000	0.000	0.000	0.000
151	0.020	0.018	0.000	0.000	0.035	0.033	0.000	0.000
157	0.000	0.107	0.000	0.050	0.121	0.033	0.000	0.050
169	0.000	0.000	0.000	0.017	0.000	0.017	0.000	0.000
Mb_Tri 1								
107	0.000	0.018	0.044	0.000	0.000	0.000	0.000	0.000
113	0.000	0.839	0.882	0.900	0.931	0.817	0.948	0.862
116	0.702	0.071	0.015	0.017	0.052	0.000	0.000	0.000
119	0.043	0.071	0.059	0.083	0.017	0.183	0.052	0.138
122	0.096	0.000	0.000	0.000	0.000	0.000	0.000	0.000
125	0.160	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mb_Tri2								
138	0.000	0.143	0.206	0.283	0.300	0.383	0.207	0.333
144	0.958	0.554	0.662	0.533	0.583	0.483	0.776	0.617
145	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
147	0.000	0.286	0.118	0.167	0.067	0.083	0.017	0.017
150	0.031	0.018	0.015	0.017	0.050	0.033	0.000	0.033
153	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.000
Mb_di5								
83	0.000	0.000	0.059	0.167	0.250	0.172	0.103	0.100
85	0.010	0.018	0.162	0.017	0.000	0.000	0.000	0.000
87	0.000	0.071	0.000	0.000	0.067	0.052	0.069	0.067
89	0.000	0.089	0.000	0.033	0.033	0.000	0.000	0.000
91	0.500	0.446	0.427	0.433	0.317	0.328	0.431	0.417
93	0.000	0.143	0.000	0.083	0.067	0.035	0.000	0.017
95	0.398	0.161	0.059	0.067	0.200	0.259	0.224	0.200
97	0.010	0.054	0.088	0.067	0.050	0.121	0.069	0.167
99	0.051	0.018	0.000	0.000	0.000	0.000	0.000	0.000
101	0.031	0.000	0.132	0.017	0.017	0.035	0.103	0.033
105	0.000	0.000	0.059	0.117	0.000	0.000	0.000	0.000
113	0.000	0.000	0.015	0.000	0.000	0.000	0.000	0.000

Mb_Tetra2								
156	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
160	0.051	0.000	0.000	0.000	0.000	0.000	0.000	0.000
168	0.000	0.000	0.044	0.017	0.033	0.000	0.000	0.052
172	0.010	0.054	0.000	0.000	0.000	0.000	0.000	0.000
176	0.010	0.036	0.000	0.000	0.000	0.000	0.000	0.000
180	0.020	0.196	0.338	0.207	0.200	0.133	0.155	0.241
184	0.214	0.054	0.118	0.017	0.017	0.000	0.000	0.000
188	0.133	0.089	0.088	0.086	0.050	0.000	0.000	0.000
192	0.388	0.161	0.015	0.052	0.017	0.000	0.000	0.000
196	0.092	0.071	0.044	0.017	0.017	0.067	0.000	0.000
200	0.071	0.018	0.029	0.035	0.050	0.033	0.017	0.017
204	0.010	0.071	0.162	0.310	0.300	0.283	0.379	0.293
208	0.000	0.071	0.000	0.121	0.217	0.383	0.259	0.276
212	0.000	0.036	0.132	0.121	0.033	0.100	0.172	0.035
220	0.000	0.036	0.000	0.000	0.017	0.000	0.000	0.000
224	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
228	0.000	0.018	0.000	0.017	0.050	0.000	0.017	0.086
232	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
240	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
248	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
252	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000
Mb_Tri 3								
127	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.000
133	0.684	0.857	0.794	0.767	0.776	0.867	0.857	0.900
142	0.306	0.089	0.162	0.183	0.121	0.133	0.107	0.100
148	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000
154	0.000	0.054	0.044	0.050	0.069	0.000	0.036	0.000
Mb_Tri 4								
99	0.796	0.446	0.221	0.317	0.283	0.133	0.138	0.233
102	0.204	0.214	0.368	0.250	0.250	0.600	0.603	0.550
108	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.000
111	0.000	0.268	0.294	0.367	0.400	0.217	0.241	0.183
114	0.000	0.071	0.088	0.067	0.067	0.050	0.017	0.033

ABSTRACT

ABSTRACT

Ringkasan

Ikan pelangi (*Melanotaeniidae*) didistribusikan antara New Guinea dan Australia. Mereka sangat dicari oleh hobiis akuarium karena karena warna mereka yang luar biasa bagus. Sejumlah besar spesies, berada di daftar merah spesies terancam punah. Spesies *Melanotaenia boesemani*, adalah salah satu spesies yang paling populer pada famili *Melanotaenia* dan sudah terancam punah. Budidaya spesies ini muncul sebagai solusi yang menjanjikan untuk membatasi penangkapan di habitat alam. Namun jumlah peternakan penangjaran *M. boesemani* sangat rendah. Ini mungkin karena masalah yang dihadapi oleh pembudidaya, yaitu proporsi yang lebih tinggi pada betina, hilangnya warna, tingkat pertumbuhan dan kesuburan rendah, serta sering terjadinya cacat morfologi. Dalam konteks disertasi ini, adalah untuk menghasilkan data genetik baru dalam meningkatkan budidaya dan konservasi famili *Melanotaenia*. Secara khusus, tujuan adalah: 1) untuk mengembangkan penanda mikrosatelit baru dari DNA *M. boesemani*, 2) menilai keragaman genetik populasi liar dari *Melanotaenia* dan memperbaiki taksonomi mereka, 3) untuk menentukan asal geografis dari strain *M. boesemani* yang telah dibudidayakan di Indonesia, dan untuk menilai tekanan inbreeding yang dihasilkan dari perkawinan sedarah pada domestikasi ini. Untuk sekuensing, sebanyak 12 penanda mikrosatelit dikembangkan dan divalidasi pada spesies *M. boesemani*. Lokus yang sesuai semua ditemukan polimorfik dan percobaan crossing telah menunjukkan bahwa mereka mematuhi hukum Mendel.

Penanda baru yang kemudian digunakan untuk mengevaluasi keragaman genetik 44 populasi liar (sesuai dengan 1.152 spesimen ikan). Nilai-nilai Fis multilokus mengungkapkan bahwa 5 spesies menunjukkan perbedaan yang signifikan dalam keseimbangan dan Hardy-Weinberg menyarankan kemungkinan adanya subpopulasi dibedakan secara genetik. Dikombinasikan dengan analisis filogenetik dari sitokrom oksidase gen I (IOC) dan pengamatan beberapa karakter morfologi diagnostik, 12 penanda mikrosatelit juga digunakan untuk mengkarakterisasi 8 spesies baru yang belum teridentifikasikan. Akhirnya, penanda mikrosatelit yang diterapkan untuk menganalisa dan membandingkan variabilitas genetik *M. boesemani* diperoleh dari enam peternakan sekitar Jakarta dengan dua populasi asli dari danau Ayamaru dan danau Uter (Papua Barat). Hasil penelitian menunjukkan bahwa semua strain yang telah di budidayakan berasal dari danau Ayamaru. Tidak ada kekurangan heterozigot kekurangan yang teramati, menunjukkan bahwa tidak ada perkawinan sedarah yang signifikan dalam strain budidaya tersebut. Analisis genotipe juga menunjukkan bahwa spesies *M. boesemani* yang dibudidayakan merupakan populasi yang berbeda. Pada akhirnya, hasil ini menunjukkan bahwa masalah yang dihadapi oleh pembudidaya ikan bukan karena perkawinan sedarah mungkin tapi yang paling mungkin terkait dengan faktor-faktor lain seperti manajemen budidaya yang tidak memadai dan / atau perairan pembibitan yang berkualitas buruk. Kesimpulannya, penanda mikrosatelit baru ini berguna untuk mengevaluasi struktur genetik dan keragaman spesies ikan pelangi, yang banyak mulai terancam punah. Hasil yang disajikan di sini di salah satu spesies paling terancam punah (*M. boesemani*) menunjukkan bahwa masih mungkin untuk mencegah kepunahan. Budidaya merupakan solusi untuk menekan penangkapan dihabitat alami. Ini membutuhkan manajemen yang lebih baik pada praktek pembudidayaan.

Kata kunci: Budidaya, keanekaragaman genetik, mikrosatelit, Ikan pelangi, *Melanotaenia boesemani*, Spesies baru *Melanotaenia*, Filogeni, Papua Barat.

Résumé

Les poissons arc-en-ciel (*Melanotaeniidae*) se distribuent entre la Nouvelle-Guinée et l'Australie. Ils sont très recherchés en aquariophilie en raison de leur coloration remarquable. Il en existe un très grand nombre d'espèces, dont certaines figurent sur la liste rouge des espèces menacées. L'espèce *Melanotaenia boesemani*, l'une des plus populaires au sein de cette famille, est en voie de disparition. L'aquaculture de cette espèce apparaît donc comme une solution prometteuse pour limiter la capture de spécimens sauvages. Pourtant, le nombre de fermes qui élèvent *M. boesemani* est très faible. Ceci est probablement dû aux problèmes rencontrés par les aquaculteurs, à savoir une proportion plus élevée de femelles par ponte, une perte de la coloration, un taux de croissance et une fécondité plus faibles, ainsi que l'apparition fréquente de malformations. Dans ce contexte, cette thèse visait à produire de nouvelles données génétiques en vue d'améliorer l'aquaculture et la conservation de cette famille. Plus précisément, les objectifs étaient: 1) de développer de nouveaux marqueurs microsatellites à partir d'ADN de *M. boesemani*, 2) d'évaluer la diversité génétique des populations sauvages de *Melanotaenia* et d'affiner leur taxonomie, 3) de définir l'origine géographique des souches de *M. boesemani* élevées en Indonésie, et d'évaluer la pression de consanguinité résultant de cette domestication. Par séquençage haut débit, 12 marqueurs microsatellites ont été développés et validés sur l'espèce *M. boesemani*. Les loci correspondant se sont tous révélés polymorphe et des expériences de croisement ont montré qu'ils se conformaient aux lois de Mendel.

Ces nouveaux marqueurs ont ensuite été mis en œuvre pour évaluer la variabilité génétique de 44 populations sauvages (correspondant à 1.152 spécimens de poissons). Les valeurs de Fis multilocus ont révélé que 5 espèces présentaient des écarts significatifs à l'équilibre de Hardy-Weinberg et suggéré la présence possible de sous-populations génétiquement différenciées. Combinés à une analyse phylogénétique effectuée sur le gène de la cytochrome oxydase I (COI) et à l'observation de plusieurs caractères morphologiques diagnostic, les 12 marqueurs microsatellites ont également permis de caractériser 8 nouvelles espèces non-encore décrites. Enfin, ces marqueurs microsatellites ont été appliqués pour analyser et comparer la variabilité génétique d'échantillons de *M. boesemani* obtenus à partir de 6 fermes aquacoles autour de Jakarta avec celle des deux populations indigènes de cette espèce, à savoir des lacs Ayamaru et Uter (Papouasie occidentale). Les résultats ont indiqué que toutes les souches élevés provenaient du lac Ayamaru. Aucun déficit en hétérozygotes n'a été mis en évidence, suggérant qu'il n'y avait pas de consanguinité majeure dans ces souches d'élevage. L'analyse des génotypes a également suggéré que l'espèce *M. boesemani* représentait probablement une métapopulation constituée de populations génétiquement différenciées. En définitive, ces résultats indiquaient que les problèmes rencontrés par les aquaculteurs ne proviennent pas d'une éventuelle consanguinité mais sont plus surement liés à d'autres facteurs tels qu'une gestion inappropriée et / ou une mauvaise qualité des eaux d'élevage. En conclusion, ces nouveaux marqueurs microsatellites se sont avérés utiles pour évaluer la structure génétique et la diversité d'un grand nombre d'espèces de poisson arc-en-ciel, dont beaucoup sont en voie de disparition. Les résultats présentés ici sur l'une des espèces les plus menacées (*M. boesemani*) montrent qu'il est encore possible d'éviter son extinction. Ceci nécessite cependant d'augmenter sa production aquacole afin de soulager rapidement la pression de surpêche. Ceci passe par une meilleure gestion des pratiques d'élevage.

Abstract

Rainbowfishes (*Melanotaeniidae*) are widely distributed throughout New Guinea and Australia. They are very famous for ornamental trade because of their vivid coloration. They display amazing species richness and some of them are on the red list of endangered species. The species *Melanotaenia boesemani*, one of the most popular within this family, is.facing great threats. Rearing of this species in aquaculture setups thus appears as a promising solution to limit capture of wild specimens. Yet, the number of farms that raise *M. boesemani* is very low. This is probably due to the problems reported by the farmers, i.e. higher proportion of females per spawning, loss of coloration, lower growth rate and fecundity, frequent morphological abnormalities. In this context, this study aimed at gathering new genetic information that would be useful for the aquaculture and conservation of the *Melanotaeniidae* family. Specifically, the objectives of the research were: 1) to develop new microsatellite DNA markers from the endangered *M. boesemani*, 2) to evaluate the genetic diversity of wild populations of *Melanotaenia* and refine their taxonomy, 3) to describe the geographic origins of *M. boesemani* reared by ornamental fish farmers in Indonesia, and evaluate the inbreeding pressure resulting from this domestication. Using next generation sequencing, 12 microsatellite DNA markers were developed and validated from *M. boesemani.* All microsatellite loci revealed polymorphic and cross-breeding experiments showed that they followed a Mendelian inheritance pattern.

These new markers were subsequently implemented to evaluate the genetic variability of 44 wild populations (corresponding to 1.152 fish specimens). Multilocus Fis values revealed that 5 species significantly departed from Hardy-Weinberg expectations and suggested the possible occurrence of genetically differentiated subpopulations. Combined with a phylogenetic analysis performed on the cytochrome oxydase I (COI) gene and with the observation of several diagnostic morphological characters, the 12 microsatellite markers also enabled to characterize 8 new species previously undescribed. Finally, these microsatellite markers were applied to analyze and compare the genetic variability of *M. boesemani* samples obtained from 6 aquaculture farms around Jakarta with that of the two native populations of this species , i.e. from Ayamaru and Uter Lakes (West Papua). Results indicated that all reared strains originated from Ayamaru Lake. No deficit in heterozygotes was evidenced, suggesting that there was no major inbreeding in these reared populations. Genotype analysis also suggested that *M. boesemani* species consists of a metapopulation composed of genetically differentiated populations. Altogether, these results indicated that the problems experienced by the farmers are obviously not due to inbreeding depression and are probably caused by other factors such as unsuitable management and/or poor water quality. In conclusion, these new microsatellite markers proved useful to evaluate the genetic structure and diversity of a large number of rainbowfish species, among which many are endangered. The results presented here on one of the most threatened species (*M. boesemani*) show that it is still possible to prevent its extinction. This, however, implies to increase its aquaculture production in order to quickly alleviate the overfishing pressure. This, in turn, involves a better management of rearing practices..

Mot clés : Aquaculture, Biodiversité génétique, Microsatellites, Les poissons arc-en-ciel, *Melanotaeia boesemani*, Nouvelles espèces *Melanotaenia*, Phylogénie, Papouasie occidentale,