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Genetic diversity of the grapevine vector nematode Xiphinema index and application to optimize the resistance strategy

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THÈSE DE DOCTORAT

Diversité génétique du nématode vecteur
Xiphinema index sur vigne et application pour
optimiser la stratégie de résistance

Van Chung NGUYEN

Unité de recherche : UMR ISA INRA1355-UNS-CNRS7254

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du grade de docteur en :**
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de l'Université Côte d'Azur

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Titre: Diversité génétique du nématode vecteur *Xiphinema index* sur vigne et application pour optimiser la stratégie de résistance

Résumé

Du fait du retrait des nématicides, des alternatives de lutte contre les espèces majeures de nématodes parasites des plantes sont urgentes et la résistance naturelle des plantes est une méthode prometteuse. En vignoble, le nématode *Xiphinema index* a une forte incidence économique en transmettant le *Grapevine fanleaf virus* (GFLV), la première virose de la vigne à l'échelle mondiale. Face à ce fléau, des porte-greffe résistants dérivés de la muscadine sont en cours de sélection chez la vigne et utilisent *Muscadinia rotundifolia* comme source de résistance au nématode vecteur, afin d'arrêter ou retarder la transmission du GFLV. Chez les plantes pérennes et la vigne en particulier, l'interaction longue entre la plante et le nématode accroît le risque de contournement de la résistance. Une étude de génétique des populations a montré que cette espèce parthénogénétique méiotique se reproduit aussi (rarement) par voie sexuée au champ, ce qui peut encore accroître ce risque. La durabilité de la résistance est donc un vrai défi qui doit prendre en compte la diversité génétique du nématode à l'échelle mondiale. Une étude phylogénétique préliminaire avait permis de révéler les clades de diversité prédominants, de sélectionner des populations représentatives et de créer, à partir de ces dernières, des lignées isofemelles utilisées dans notre étude de durabilité de résistance.

Dans ce contexte, la première partie de la thèse a confirmé et approfondi l'approche phylogénétique en s'appuyant sur une gamme élargie d'échantillons de nématodes. Cette étude basée sur des séquences mitochondriales (marqueurs maternels) et sur des locus microsatellites (marqueurs nucléaires neutres) a exploité les données de plus de 80 échantillons représentatifs de l'aire mondiale de distribution de *X. index*, qui s'étend du Moyen et Proche-Orient, à la partie Est, Centrale et Ouest du Bassin Méditerranéen, et jusqu'aux pays occidentaux (Europe et Amérique du Nord et du Sud). Le marqueur mitochondrial *CytB* a d'abord été utilisé pour comparer *X. index* avec une espèce vectrice proche mais amphimictique, *X. diversicaudatum*. *Xiphinema index* a montré une variabilité moléculaire nettement plus faible que *X. diversicaudatum*, en accord avec leurs modes de reproduction respectifs. Nous avons ensuite observé que les séquences concaténées de *CytB* et de trois autres gènes mitochondriaux *ATP6*, *ND4* et *CO1*, expriment un patron phylogéographique robuste qui définit trois clades regroupant les échantillons de l'Est Méditerranéen, et un seul clade regroupant les échantillons de Méditerranée de l'Ouest, d'Europe et des Amériques. Le polymorphisme mitochondrial le plus élevé est observé chez l'un des clades du Moyen et Proche-Orient qui englobe les Régions Transcaucasienne et Sud de la Mer Caspienne où la vigne a vraisemblablement été domestiquée et incluraient la région native du nématode. La dissémination ultérieure du nématode d'Est en Ouest durant l'Antiquité vraisemblablement par les Grecs et les Romains se superpose avec celle de sa plante-hôte 'vigne domestiquée'. En Méditerranée de l'Ouest, en Europe et en Amérique, seuls deux haplotypes mitochondriaux très proches sont détectés. Le premier, présent dans les vignobles du Sud de la Péninsule Ibérique, de Bordeaux et de Provence, montre un polymorphisme microsatellite élevé et aurait été introduit durant l'Antiquité. En revanche le second haplotype contient un génotype prédominant très largement répandu dans les pays occidentaux et nous émettons l'hypothèse que ceci est dû à sa

dispersion récente lors des replantations massives qui ont suivi la crise phylloxérique de la fin du 19^{ème} siècle.

La deuxième partie de la thèse a abordé la durabilité de la résistance en conditions contrôlées et au champ. En conditions contrôlées, l'étude a été conduite sur des matériels résistants F1 et BC1 dérivés de la muscadine, en comparant du matériel préalablement multiplié sous forme *in vitro* et du matériel issu de bouturage ligneux classique. Le facteur de reproduction des nématodes a été suivi sur les plants âgés de 3 à 6 ans inoculés avec un mélange en proportions égales de quatre lignées représentatives retenues précédemment. Nos résultats ont montré que les nématodes élevés sur du matériel issu d'*in vitro* contournent progressivement la résistance alors que le matériel issu de bouturage classique exprime une résistance durable. Pour le matériel issu d'*in vitro*, l'hypothèse d'un effet sensibilisant, consécutif à sa longue conservation en conditions *in vitro* avant son sevrage dans le sol pour la mise en place de l'étude, est mise en avant. La différence d'aptitude d'hôte entre les deux types de matériels élimine *a priori* l'hypothèse d'une adaptation génétique du nématode comme cela a été observé chez les nématodes à galles *Meloidogyne* spp. vis-à-vis des Solanacées. Par l'analyse de leurs génotypes microsatellites multilocus (MLGs), des individus hybrides entre lignées différentes ont été détectés à un taux faible mais croissant entre 4 et 6 ans, quels que soient le type de multiplication et le niveau de résistance du matériel végétal. Ces résultats confirment en conditions contrôlées la possibilité de reproduction sexuée déjà observée au champ mais écartent le phénomène d'hybridation comme mode d'adaptation permettant d'aboutir à un contournement de la résistance du matériel issu d'*in vitro*. Par ailleurs, l'analyse des MLGs a aussi montré des différences d'agressivité entre les lignées en mélange même si aucune n'a été exclue ou au contraire est devenue complètement prédominante au cours des années. La multiplication progressive du nématode chez le matériel résistant issu d'*in vitro* au cours des années doit être mise en relation avec une architecture différente du système racinaire chez les plantes de ce type et pourrait être due à des modifications physiologiques discrètes mais durables des tissus racinaires apicaux aux dépens desquels le nématode se nourrit. Enfin, en complément de l'étude en conditions contrôlées, l'étude de durabilité au champ a analysé les effectifs de nématodes sur le porte-greffe résistant BC1 et sur des témoins sensibles à *X. index* dans un essai âgé de 16 ans implanté en parcelle initialement très infestée par des nématodes virulifères. A cette date, les nématodes sont quasiment indétectables sur le matériel résistant qui est dans un bon état de développement végétatif malgré les attaques virales alors que leurs effectifs sont plus élevés, tout en restant très faibles, sur les matériels témoins sensibles à *X. index* et au GFLV qui sont morts ou fortement dépérissants. Pris en compte globalement, nos résultats montrent que la stratégie de résistance au nématode utilisant du matériel multiplié par bouturage ligneux classique apparaît durable. Cette stratégie ciblée sur le contrôle du vecteur contribuera donc à réduire fortement l'impact du GFLV transmis par *X. index* en vignoble.

Mots-clés : Diversité, vigne domestiquée, durabilité, vigne, microsatellite, gène mitochondrial, *Muscadinia rotundifolia*, résistance des plantes, propagation des plantes, phylogéographie, *Xiphinema*, *X. index*, nématode vecteur

Title: Genetic diversity of the grapevine vector nematode *Xiphinema index* and application to optimize the resistance strategy**Abstract**

Because of the ban of most nematicides, control alternatives are urgently needed against plant-parasitic nematodes affecting major crops and breeding for plant varieties carrying natural resistance (R) is promising. In vineyards, the nematode *Xiphinema index* has a high economical impact by transmitting *Grapevine fanleaf virus* (GFLV), the first viral disease worldwide. Consequently muscadine-derived resistant rootstocks are being selected in grapevine, using *Muscadinia rotundifolia* as an R source to the vector, in order to arrest or delay GFLV transmission. In perennial crops and in grapevine in particular, the long plant-nematode interaction increases the risk of resistance breaking. A previous population genetics study had shown that this meiotic parthenogenetic nematode is able to reproduce sexually (rarely) in the field, which may still enhance this breaking risk. Thus, resistance durability is a real challenge that must consider the global nematode diversity. A preliminary phylogenetic work had allowed revealing the predominant diversity clades, selecting representative populations, and creating, from these latter, single-female lines to be used in our resistance durability experiments.

In this context, the first part of the thesis has confirmed and deepened our phylogeographical approach using an extended coverage of nematode samples. Our study based on mitochondrial sequences (maternal markers) and microsatellite loci (neutral nuclear markers) used more than 80 *X. index* samples representative of the worldwide nematode distribution, collected from the Middle- and Near East, the Eastern-, Central- and Western Mediterranean, and the Western countries (Europe and the Americas). The mitochondrial marker *CytB* was first considered for comparison of *X. index* with the related amphimictic vector species *X. diversicaudatum*. *Xiphinema index* exhibits a significantly lower intraspecific molecular variability than *X. diversicaudatum*, in agreement with the respective reproduction modes of both nematodes. We then showed that concatenated sequences from *CytB* and three additional mitochondrial genes *ATP6*, *ND4* and *CO1*, display a robust phylogeographical pattern consisting in three clades grouping Eastern Mediterranean, Near- and Middle Eastern samples and a single clade grouping samples from Western Mediterranean, Europe and the Americas. The highest mitochondrial polymorphism is observed in one of the clades from the Middle- and Near-East that overlaps the Transcaucasian and Southern Caspian Sea regions from where grapevine has been presumably domesticated and that likely overlaps the nematode native area. East-to-west nematode dissemination appears to match that of its 'domesticated grapevine' host during the Antiquity mainly by the Greeks and then the Romans. In Western Mediterranean, Europe and the Americas, only two close mitochondrial haplotypes are detected. The first haplotype, found in vineyards from the Southern Iberian Peninsula, Bordeaux and Provence, exhibits a high microsatellite polymorphism presumably due to introductions during the Antiquity. By contrast, the second haplotype contains a highly

predominant microsatellite genotype surprisingly widespread in most Western countries and we hypothesize that its wide distribution is consecutive to its recent dispersal during the massive grapevine replants following the 19th-century phylloxera crisis.

The second part of the thesis performed an evaluation of the durability of muscadine-derived rootstock material under both controlled and field conditions. Under controlled conditions, the durability study was performed with F1 and BC1 resistant rootstock material previously obtained from either *in vitro* or hardwood-cutting propagation. The nematode reproduction factor has been monitored in plants aged 3 to 6 years inoculated with a mix, in equal proportions, of four *X. index* representative lines previously retained. Our results have established that nematodes from plants obtained from *in vitro* progressively overcame the resistance while the material obtained from cuttings showed a durable resistance. For material obtained from *in vitro*, the hypothesis of a 'susceptibilization' effect, consecutive to the long conservation of the resistant stock material in *in vitro* culture before its acclimatization in the soil for the durability experiment, is proposed. The contrasting behavior between the two propagation types *a priori* removes the hypothesis of a nematode genetic adaptation as described, e.g., for root-knot nematodes *Meloidogyne* spp. towards Solanaceous crops. By analyzing microsatellite multilocus genotypes (MLGs), hybrid individuals between different lines have been detected at a low but increasing rate between 4 and 6 years, whatever the propagation type and the resistance status. These results confirm under controlled conditions the ability of sexual reproduction already observed in the vineyard but discard the hybridization phenomenon as the mode of adaptation of the nematode that leads to the resistance breakdown from *in vitro* plants. Moreover, MLGs analysis also showed that differences in aggressiveness between mixed lines appeared, even though none of them was eliminated or conversely became completely predominant over years. Nematode progressive multiplication in R accessions obtained from *in vitro* appears linked to a different architecture of the root system in this propagation type that may have induced discrete but putatively durable physiological changes in apical root tissues from where nematodes feed. The complementary durability study in field conditions has analyzed the nematode numbers on the BC1 resistant rootstock and on susceptible rootstocks from an experiment aged 16 years, settled in a plot initially highly infected with viruliferous nematodes. At that date, nematodes have been almost undetectable on the resistant BC1 rootstock, which remained in a good vegetative developmental state despite GFLV attacks, while higher, though very low, numbers have been detected on susceptible rootstocks that were dead or in advanced decline. Taken all together, our results show that the muscadine-derived resistance strategy, using classical hardwood cutting material, appears durable. This method focused on vector control will significantly contribute to reduce the impact of GFLV transmitted by *X. index*.

Key words: diversity, domesticated grapevine, durability, grapevine, microsatellite, mitochondrial gene, *Muscadinia*, plant resistance, plant propagation, phylogeography, *Xiphinema*, *X. index*, vector nematode

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List of abbreviations

AILV	<i>Artichoke Italian latent virus</i>
ArMV	<i>Arabidopsis mosaic virus</i>
ATP6	<i>ATP synthase subunit 6</i>
CO1	<i>Cytochrome c oxidase I</i>
CS	Cabernet-Sauvignon
<i>CytB</i>	<i>Cytochrome B</i>
ELISA	Enzyme-linked immunosorbent assay
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
GCMV	<i>Grapevine chrome mosaic virus</i>
GFLV	<i>Grapevine fanleaf virus</i>
HLR	Hypersensitive-like reaction
HR	Hypersensitive response
IGGP	The International Grape Genome Program
ITS	Internal transcribed spacer
J2	Second-stage juvenile
MAMPs	Microbial-associated molecular patterns
NCBI	National Center for Biotechnology Information
NCPN	National Grape Clean Plant Network
<i>ND4</i>	<i>NADH dehydrogenase subunit 4</i>
NLRs	Nucleotide-binding leucine-rich repeat receptors
ONIVINS	Office National Interprofessionnel des Vins
PAMP	Pathogen-associated molecular patterns
PCN	Potato cyst nematode
PCR	Polymerase chain reaction
PGIPs	PG-inhibiting proteins
PPECP	Canadian Plant Protection Export Certification Program
PPN	Plant parasitic nematode
PRMV	<i>Peach rosette mosaic virus</i>
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity

QR	Quantitative resistance
RF	Reproduction factor
RGM	<i>V. riparia</i> Gloire de Montpellier
RKN	Root-knot nematode
RL	<i>V. rupestris</i> du Lot
ROS	Reactive oxygen species
RPG1	Némadex Alain Bouquet (Némadex AB)
RpRSV	<i>Raspberry ringspot virus</i>
SLRSV	<i>Strawberry latent ringspot virus</i>
SSU	Small subunit
TBRV	<i>Tomato black ring virus</i>
ToRSV	<i>Tomato ringspot virus</i>
TRSV	<i>Tobacco ringspot virus</i>
UC Davis	University of California, Davis
VR	<i>V. vinifera</i> x <i>M. rotundifolia</i>
VRH	<i>Vitis-rotundifolia</i> hybrid

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Introduction

Grapevine is one of the most economically important crops worldwide by its products i.e. mainly wine but also table grape, grape juice and raisins. Since the Antiquity, it has known successive growth and crisis phases until today and it is now gaining development in new countries. As many ancient crops, it has to face a very wide and increasing array of pests and diseases. Among these pests, plant-parasitic nematodes (PPNs) have a high impact by their attacks on the roots. Major PPNs are the highly polyphagous root-knot nematodes (RKNs), *Meloidogyne* spp., and the more specific dagger nematode *Xiphinema index*, the vector of *Grapevine fanleaf virus* (GFLV). GFLV is the first grapevine virus by its impact at the global scale. The vector nematode is present in all major world vineyards and is mainly recovered in grapevine fields with a long history of grape cultivation. Besides GFLV grape prophylaxis, control of the vector nematode is the more common strategy to limit virus damage. It used to rely on preplant soil treatments with chemical nematicides combined with fallow crops between two successive grapevines. Nevertheless, nematicides are the most toxic active ingredients used in agriculture and, because of this environmental impact, they are being progressively removed from the market. Fallow strategy requires a very long interval of at least seven years for nematode eradication and this makes it economically unacceptable in premium vineyards. Consequently, alternative nematode management strategies are urgently needed in GFLV infected vineyards.

New management strategies are challenged by biological characteristics of the nematode. The nematode is preferentially located in deep soil layers and this is an important limiting factor for its control. Another major limitation is the high affinity between the virus and its vector and the correlative long retention time of GFLV by the nematode in the soil during the fallow period separating two successive vine crops. Against these constraints, selecting grapevine material carrying germplasm resistance to the nematode and using it for breeding nematode-resistant grapevine rootstocks constitute a promising alternative as it may allow to delay significantly viral transmission. However, in grapevine as in other woody perennial crops occupying the land for several decades, the extended plant-nematode interaction is undoubtedly a major drawback by increasing the risk of resistance breaking, and resistance needs to be evaluated over extents that fit with this long plant life.

In general, plant resistance to ectoparasitic migratory nematodes has been poorly studied in comparison with sedentary endoparasitic nematodes. Nevertheless, in grapevine evaluations performed under field and controlled conditions have early illustrated that host suitability of *Vitis* species to *X. index* is variable. Clonal accessions from *V. riparia*, *V. rotundifolia* or *V. arizonica* have shown their higher resistance within *Vitis* spp. Parallel studies had also searched for resistance in close genera and accessions within *Muscadinia* material has been reported as carrying a higher level of resistance than *Vitis* spp. Muscadine grapes (*M. rotundifolia*) are now recognized as the best potential sources for resistance to *X. index*. Consequently, muscadine-derived resistant rootstocks are being selected in grapevine

in order to arrest or delay GFLV transmission. Among them, some *Vitis x Muscadinia* hybrids obtained in France display a high level of resistance but unfortunately major cultural defects inherited from their muscadine parent which render necessary at least one supplemental back cross step. This breeding scheme has been followed to select recently the rootstock 'Nemadex Alain Bouquet' to be used in plots highly infected by the nematode. Considering that this rootstock appears adapted to a limited range of edapho-climatic conditions, new rootstocks are needed to cope notably with the diversity of environments throughout the extensive grapevine geographic distribution.

Few resistance sources are available in muscadine vines. The source from where 'Nemadex Alain Bouquet' has been bred appears particularly interesting for future selection projects. Additional resistance studies are needed to characterize fully the advantages and the limits of the strategy based on this source. This knowledge will allow the integration of the resistance within new management strategies in order to guarantee a sustainable control. Optimizing this resistance strategy needs i) to consider in priority the global diversity of the nematode vector and ii) to take it into account for studies aimed at deciphering both the spectrum and the durability of resistance.

A first appraisal of the nematode genetic diversity of *X. index* has been obtained through a population genetics study at the field and farm scales. It showed that this meiotic parthenogenetic nematode is also able to reproduce sexually (rarely) in the field. A preliminary phylogenetic work had allowed to reveal the predominant diversity clades and to select representative populations to be used in resistance experiments. In this context, the thesis has a first objective that is to confirm and deepen this phylogeographical approach using an extended geographic coverage of the worldwide nematode distribution.

A consistent assessment of the spectrum and durability conferred by the muscadine source is also indispensable. Characterizing the plant host suitability to *X. index* leans on both short- and long-duration tests. Short durations allow discrimination of highly susceptible plant material before nematode numbers drop down because of plant damage but, amongst resistant material, longer durations are required to separate early resistance responses from those that are more durable. Notably such long evaluations are essential to discriminate the false (or provisional) resistance resulting from poor root development, from truly resistant material that expresses a sustainable antagonistic effect to nematode multiplication. The second objective of the PhD project is to perform a reliable evaluation of the spectrum and durability of muscadine-derived rootstock material. In conjunction with the perennial status of its grapevine host plant, the ability of *X. index* to undergo sexual reproduction, even if it is rare, theoretically enhances the risk that the resistance is broken. In this second part, the durability study will be conducted with F1 and BC1 resistant rootstock material. Results should establish whether the nematode is able to adapt to resistance over a long duration post-inoculation and, in the positive case, how this adaptation may occur. Taken together these data will contribute to optimize the strategy for resistance to the vector nematode *X. index*.

CHAPTER 1

GENERAL INFORMATION, WORK BASES AND OBJECTIVES

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CHAPTER 1: GENERAL INFORMATION, WORK BASES AND OBJECTIVES

I.1. THE NEMATODES

I.1.1. The phylum Nematoda

The nematodes are roundworms that belong to the phylum Nematoda. They have successfully adapted to nearly every ecosystem on the earth, from marine and fresh water to soil, and from the polar regions to the tropics, as well as from the highest to the lowest altitudes. They are found in every part of the earth's lithosphere (Borgonie et al., 2011). The total number of nematode species has been estimated to be about 1 million (Lambshhead, 1993). So far, more than 25,000 species have been described (Zhang et al., 2011; Zhang, 2013) of which more than half are parasitic nematodes.

The many parasitic forms include pathogens in most plants and animals as well as humans. In natural or cultivated soils, the nematofauna comprises several feeding types. These are mainly bacterivores, fungivores, omnivores, herbivores and predators. Nematodes are classified along with insects and other moulting animals in the clade Ecdysozoa, and, unlike flatworms, have tubular digestive systems with openings at both ends.

I.1.2. The plant parasitic nematodes

Among the phylum Nematoda, more than 4,100 species of plant parasitic nematodes (PPNs) have been described (Decraemer et al., 2006) causing an economical damage of approximately \$US 80 billion per year (Nicol et al., 2011). Most PPNS are soil-borne root pathogens and only a few species feed primarily upon shoot tissues. PPNS have appeared independently in four clades: Triplonchida (clade 1), Dorylaimida (clade 2), Panagrolaimoidea (clade 10) and Tylenchida (clade 12) (Fig. 1.1).

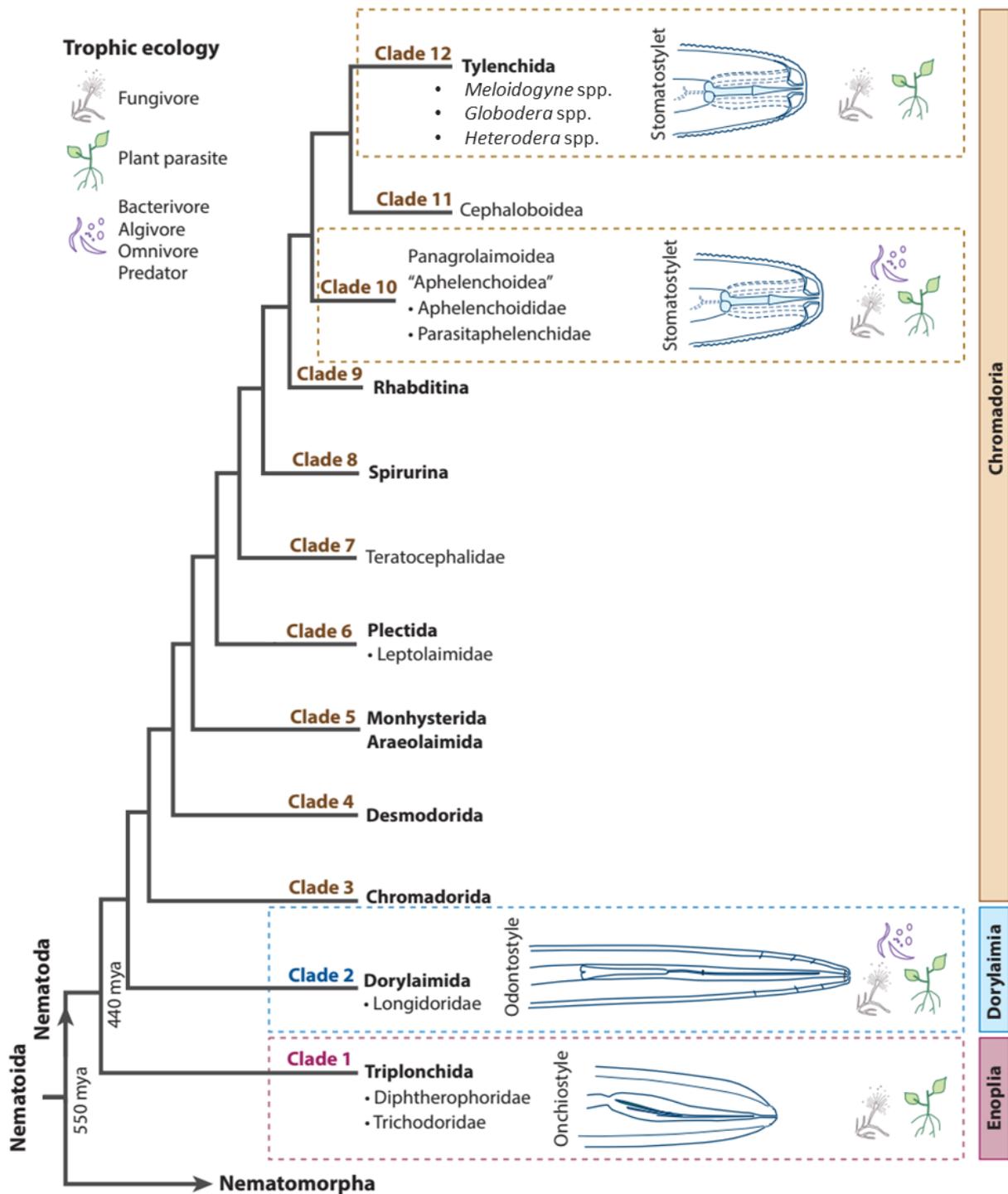


Figure 1.1. Schematic overview of the phylum Nematoda. Division in 12 major clades is based on SSU rDNA sequence data. Next to the individual branches of each clade number, the order or suborder names (with the endings -ida and -ina, respectively) are reported. Trophic ecologies are visualized by icons, and only the 4 clades containing plant parasitic nematodes are shown (clades 1, 2, 10 and 12) (adapted from Quist et al., 2015)

1.1.2.1. Biology and ecology

Plant parasitic nematodes are obligate parasites that display very diverse feeding strategies and can be classified in several types by being either sedentary or migratory and either endo- or ectoparasites (Fig. 1.2)

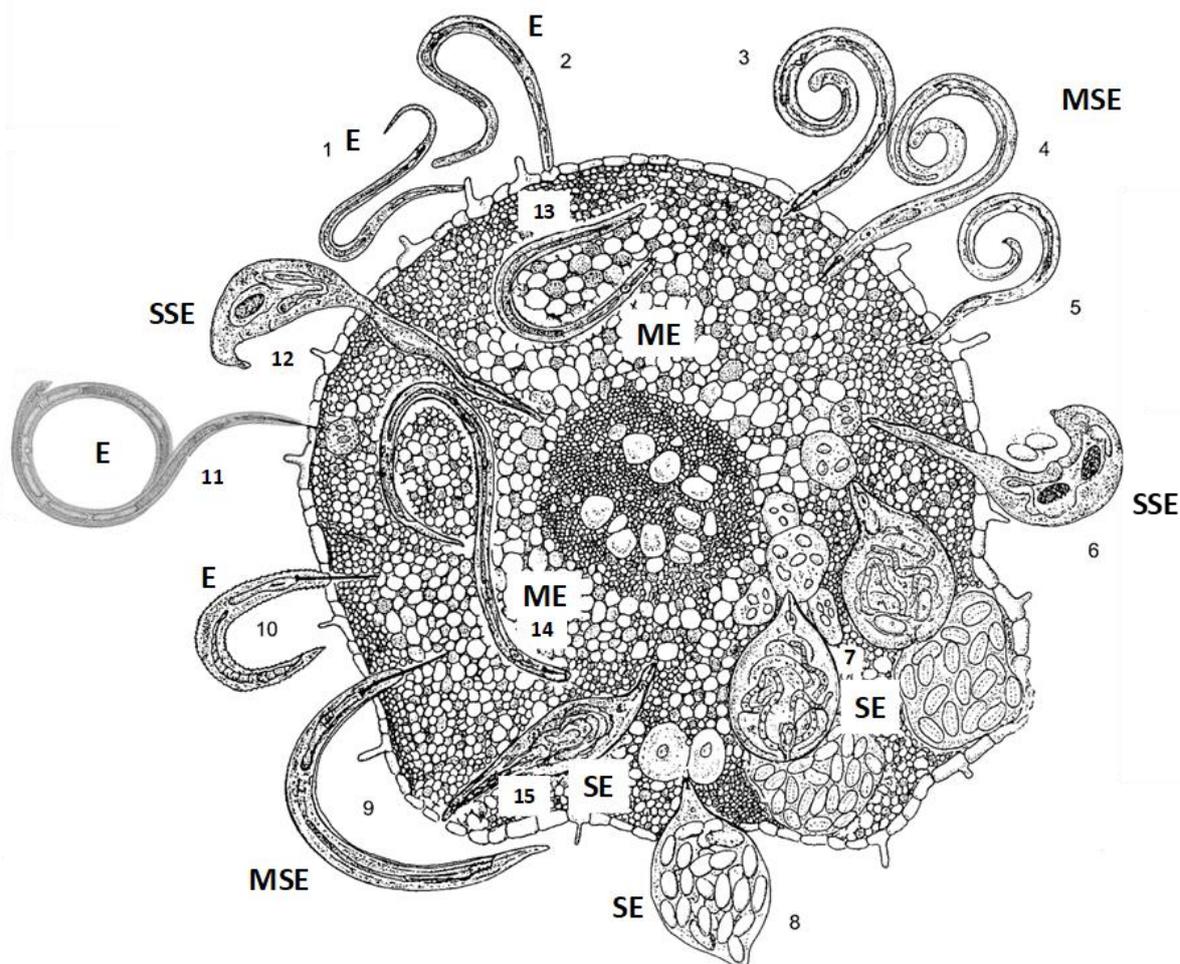


Figure 1.2. Different types of feeding strategies used by genera of plant parasitic nematodes in plants during parasitism of root tissue. Ectoparasites (E): *Ditylenchus* (1), *Tylenchorhynchus* (2), *Criconemoides* (10) and *Xiphinema* (11). Sedentary endoparasites (SE): *Meloidogyne* (7), *Heterodera* (8) and *Nacobbus* (15). Migratory endoparasites (ME): *Pratylenchus* (13) and *Hirschmanniella* (14). Migratory semi-endoparasites (MSE): *Rotylenchus* (3), *Hoplolaimus* (4), *Helicotylenchus* (5) and *Hemicycliophora* (9). Sedentary semi-endoparasites (SSE): *Rotylenchulus* (6) and *Tylenchulus* (12) (adapted from Hunt et al., 2005)

Sedentary endoparasitic nematodes

The most damaging nematodes in the world have a sedentary endoparasitic life style. The two main groups belong to the order Tylenchida and are the root-knot nematodes (RKNs) (*Meloidogyne* spp.) and the cyst nematodes (*Heterodera* spp. and *Globodera* spp.).

Root-knot nematodes (*Meloidogyne* spp.)

The common name of this genus is due to the characteristic symptom of galls (root knots) induced by the mobile second-stage juveniles while infecting plant roots that subsequently disturb plant development and reduce its growth. This genus is prevalent at the world scale. RKNs are one of the three most economically damaging genera of plant-parasitic nematodes on protected and open field crops and are distributed worldwide. The species with the highest economic impact are parthenogenetic and are able to parasitize a very wide range of plants (Sasser et al., 1984). In the genus *Meloidogyne*, more than 97 species have been described (Hunt & Handoo, 2009). Four main *Meloidogyne* species (*M. javanica*, *M. arenaria*, *M. incognita* and *M. hapla*) are major pests worldwide. A few emerging species (e.g. *M. enterolobii* and *M. chitwoodi*) are also gaining interest by their increasing economical impact (Castagnone-Sereno et al., 2013).

Meloidogyne spp. have six life stages: the egg, the four juvenile stages (J1–J4) and the adult. They are separated by five moults, the first moult (J1) occurring within the egg. Eggs grouped in egg masses are the survival stage in the soil or in root debris. Second-stage juveniles or J2s are the sole mobile stage and hatch from eggs to penetrate into the plant. The free phase in the rhizosphere of the host plant is very short because J2s have poor energetic reserves. After penetration, J2s migrate between cortical cells of the root elongation region until the central cylinder in which they settle to undergo their sedentary phase. To become sedentary, they select a few cells of the stele to feed from, thus forming a ‘feeding site’, grouping several ‘giant cells’. In this endophytic sedentary phase, they undergo three molts to become adults. When nematode density is high in root tissues and limits feeding, abundant numbers of males may appear. In the mitotic parthenogenetic prevalent species (*M. arenaria*, *M. incognita* and *M. javanica*), progenies are indeed clones, as males do not participate to reproduction and their occurrence only regulates nematode populations. In meiotic parthenogenetic species such as *M. hapla* or *M. chitwoodi*, males, by mating with females, may induce the production of hybrid progenies. The life cycle extends from one to three months, depending on the temperature, and many hundreds of eggs can be produced per female and grouped in a single egg mass (Castagnone-Sereno et al., 2013) (Fig. 1.3).

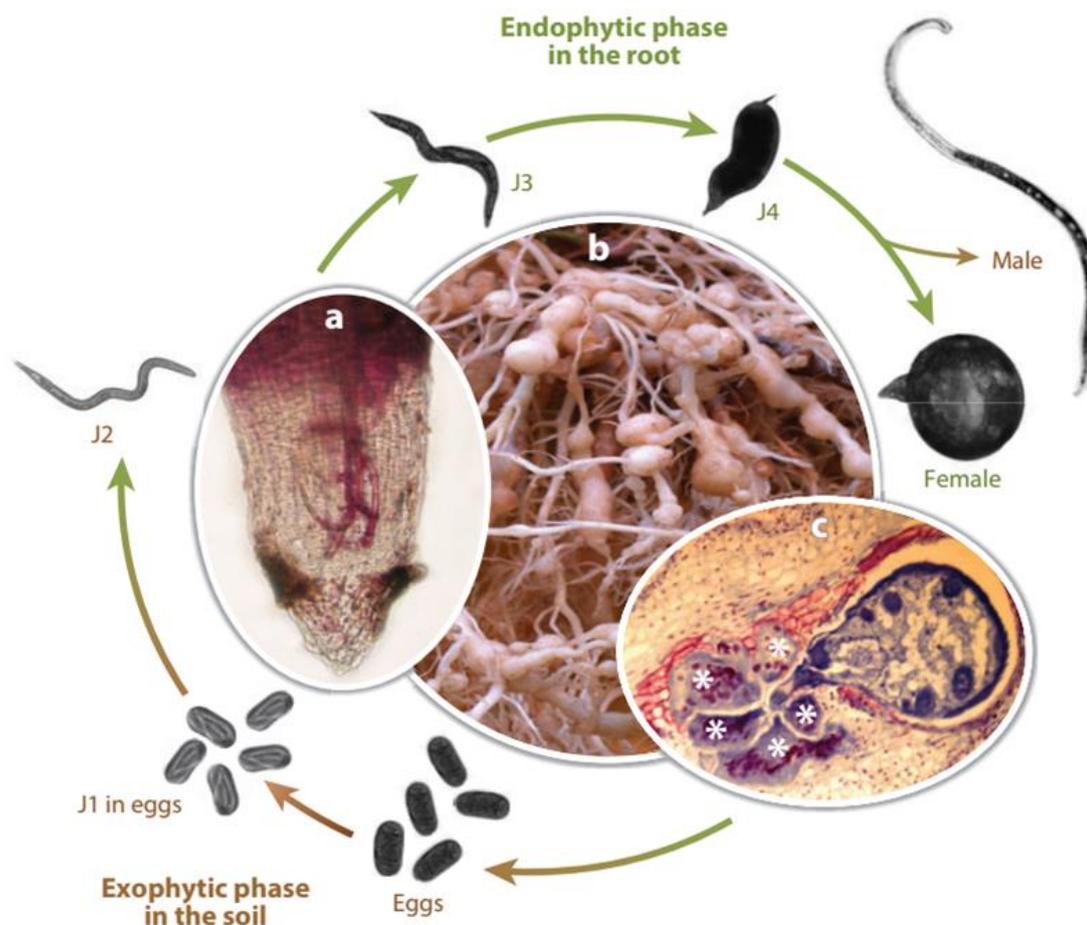


Figure 1.3. Life cycle of a mitotic parthenogenetic RKN. (a) A root tip with J2s inside. (b) Typical symptoms (i.e. galls) on tomato roots. (c) An infested root showing a female and five giant cells (*) constituting the nematode feeding site (Castagnone-Sereno et al., 2013)

Cyst nematodes (*Heterodera* spp. and *Globodera* spp.)

These nematodes belong to the Family Heteroderidae and are unique among PPNs because of the ability of the female to transform into a tough cyst that protects the eggs. The "cyst" is the body of a dead female nematode containing hundreds of eggs. Cysts can persist with viable eggs in the soil for many years, withstanding drought and frost (Stone, 1979) and remaining resistant to chemical and biological stresses. Cyst nematodes contain the two major genera *Heterodera* and *Globodera* which induce severe damage to the roots and cause growth retardation and early plant senescence at high population densities.

As RKNs, cyst nematodes have six life stages (egg, four juvenile stages, and adult). Most species have both male and female adult forms and reproduce sexually. As J1s remain in the egg envelope, J2s hatch directly into the soil and migrate on short distances towards host plant roots. After penetration, they reach the stele tissues by migrating intracellularly, contrary to RKNs. They become sedentary and induce a syncytium fusing several cells on which they feed. They complete their life cycle by molting three times until the adult stage (Jones & Northcote, 1972) (Fig. 1.4).

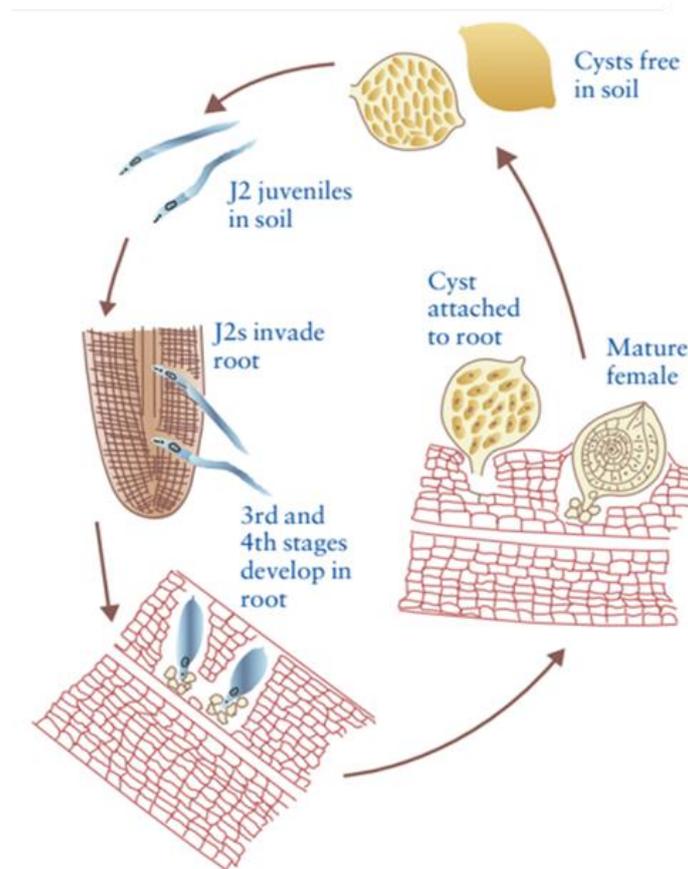


Figure 1.4. Life cycle of cyst nematodes *Heterodera* spp. and *Globodera* spp. (Bridge & Starr, 2007)

Migratory endoparasitic nematodes

These nematodes spend much of their life cycle migrating through root tissues, destructively feeding on plant cells. Major genera are *Pratylenchus*, *Radopholus* and *Hirschmanniella* (Fig.1.2) Furthermore, these nematodes may cause extensive wounds in plant roots, that lead to a potential secondary infection by bacteria and fungi (Zunke, 1990). They live freely in the soil and lay eggs within the root tissue. Nematodes from the genus *Pratylenchus* are known commonly as lesion nematodes. They are responsible for root lesion disease on many taxa of host plants in temperate regions around the world. Lesion nematodes usually only feed on the cortex of the roots. Root lesion nematodes infect a great variety of hosts. *Pratylenchus penetrans* alone has over 350 host plants and its distributed widely throughout temperate areas of the world (Corbett, 1973). Root lesion nematodes can cause significant yield losses.

Semi-endoparasitic nematodes

During their life cycle, nematodes that behave as semi-endoparasites are able to penetrate partially at different points of the root system for feeding. They are either migratory, e.g. the genera *Rotylenchus*, *Hoplolaimus*, *Helicotylenchus* and *Hemicycliophora*, or sedentary, e.g. the genera *Rotylenchulus* and *Tylenchulus*. The most economically-

important species belong to this latter group and are *Rotylenchulus reniformis* and *Tylenchulus semipenetrans* (Fig. 1.2)

Ectoparasitic nematodes

In this feeding type, nematodes remain outside the plant and use their stylet to feed from the cells of the roots. Ectoparasitic nematodes can be divided into two main groups in function of the type of damage that they cause. The first group, assembling most genera found in cultivated soils, contains species that are only responsible for direct plant damage. The second group contains a few genera that cause both direct and indirect plant damage by transmitting plant viruses.

Ectoparasitic nematodes causing only direct damage

These nematodes belong to several common genera often present in crop soils such as *Ditylenchus*, *Tylenchorhynchus* and *Criconemoides*. Plants infested with high densities in the soil may become stunted, turn yellow and produce reduced yields. Some *Xiphinema* species like *X. vuittenezi* and *X. italiae* form small galls on their host root system during their feeding and also belong to this group of nematodes.

Ectoparasitic nematodes causing direct and indirect damage

Besides their direct feeding from the plant roots, some nematode species vector plant viruses. Virus vector nematodes belong to two orders. The first order, Triplonchida (Fig. 1.1), includes the genera *Trichodorus* and *Paratrichodorus* (stubby root nematodes) that transmit tubular viruses belonging to the genus *Tobravirus* (type species *Tobacco rattle virus*) of the family Virgaviridae. The second order, Dorylaimida, includes the genera *Xiphinema* (dagger nematodes) (Fig. 1.1), *Longidorus* and *Paralongidorus* (needle nematodes), all from the family Longidoridae, that transmit viruses from the genus *Nepovirus* of the family Secoviridae. Because of their ability to transmit viruses, low nematode numbers in these species can be extremely damaging to plants (Hewitt et al., 1958; Fisher & Raski, 1967). The family Longidoridae comprises the longest plant-parasitic nematodes as their size ranges up-to 12 mm (Hooper, 1975).

Ectoparasitic nematodes have the simplest life cycle among PPNs (Fig. 1.5). Typically, eggs are laid individually in the soil, hatch at the J2 stage and all larval stages (J2-J4) and adults are parasitic. Root feeding nematodes with short stylets feed on the epidermal and outer cortical cells often at the root tips; those with longer stylets feed deeper in the cortex. Their mode of reproduction may be sexual or asexual (Bridge & Starr, 2007).

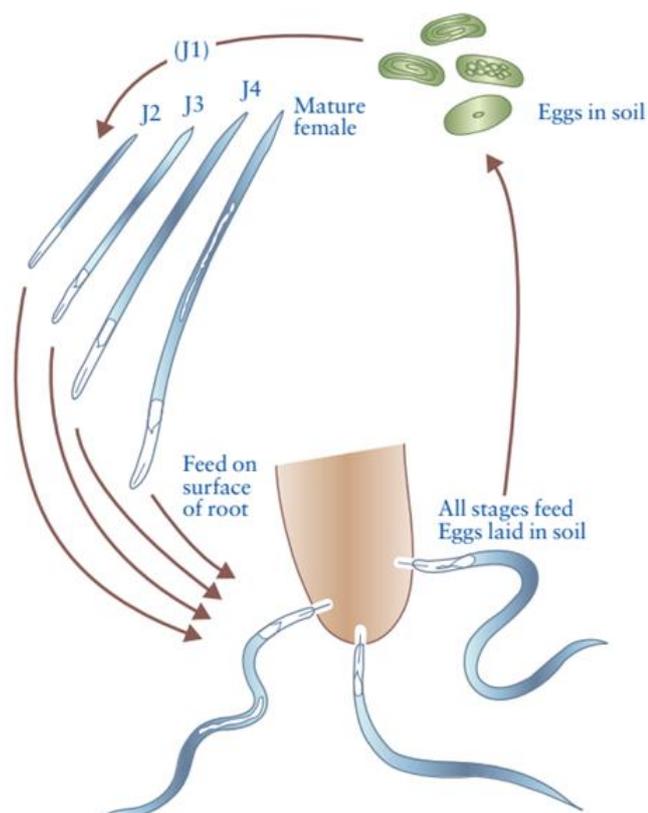


Figure 1.5. The life cycle of species from the Longidoridae family (Bridge & Starr, 2007)

The genus *Xiphinema* is of economic importance on grape, strawberry, raspberry, hops and a few other crops. Major nematodes are the vectors species of either European origin, *X. index* and *X. diversicaudatum*, or of American origin such as *X. americanum*, *X. californicum*, *X. rivesi* and *X. bricolensis*. *Xiphinema* spp. have been shown to induce moderate to large amounts of root damage through root penetration, which in most species results in the formation of galls. Efforts to study these nematodes in more details have proved problematic in some species due to difficulties in maintaining populations in a greenhouse environment. Virus vector nematodes of the genus *Xiphinema* may be associated with other non-vector *Xiphinema* species. In European grapevines, *X. index* and *X. diversicaudatum* are often accompanied by the relatively ubiquitous species *X. pachtaicum*. Other non-vector *Xiphinema* species in grapevine soils are for example *X. italiae* in Mediterranean coastal regions and *X. vuittenezi* in regions with a more continental climate.

1.1.2.2. Control

Control of PPNs used to be mainly based on chemical nematicides. Chemical nematicides are now being banned in most developed countries because of their high acute toxicity to the humans and the environment. Therefore, alternative ways must be considered.

These are mainly i) crop rotation by using non-host plants between two susceptible crops, ii) antagonistic plants that have an adverse effect which reduces the nematode densities in the soil, iii) biocontrol based on nematode enemies or toxic plant products, and iv) plant genetic resistance. Among the previous methods, a promising control alternative appears to be the use of resistant plants.

1.1.3. Resistance to plant parasitic nematodes

A plant is considered as resistant if it has the ability to hinder the growth and/or development of the pathogen (Parlevliet, 1979). In nematology, plant resistance has been defined as the ability of host plants to reduce or prevent nematode reproduction (Trudgill, 1991).

1.1.3.1. Genetics of resistance

The resistance in a host plant can be roughly divided into two types: horizontal resistance and vertical resistance (Vanderplank, 1968). Horizontal resistance is not specific to a race, a strain, a population, or an isolate of the pathogen (or pest), and thus it is characterized by the absence of a specific interaction between the host and each pathogen (or pest) genotype. Vertical resistance is race-, strain-, population-, or isolate-specific and is characterized by a particular interaction between the genotypes of the host and the pathogen (or pest) (Vanderplank, 1968). In general, horizontal resistance is considered as polygenic and thus more durable while vertical resistance is considered as monogenic and easier to overcome.

Plants have evolved a complex defense system to resist invaders. Preformed elements of defense, such as cell walls and their reinforcements, are the first barrier for any kind of invader (Underwood, 2012) and are examples of horizontal resistance. The plant defensive arsenal also includes a broad diversity of constitutively produced toxic phytochemicals (Broekaert et al., 1997) that fit with this type of resistance. To counter these defenses, nematodes use their stylet to puncture the cell wall with physical force while simultaneously releasing a mixture of secretions into the host to aid in penetration (Bohlmann and Sobczak, 2014). Besides these, a more sophisticated system of responses is induced upon infection that is based on the capability of plants to recognize, identify and block the invader at the level of a gene (= protein) called a resistance (*R*) gene. Such a resistance based on a simple genetic basis is closer to the notion of vertical resistance as it generally controls totally the pathogen (or pest). Nevertheless, the ability of the pathogen to vary and evolve genetically may induce

its rapid evolution for adaptation to the intense selection pressure exerted by the *R* gene and may result in the resistance breaking. A breakdown in resistance is due to an increase in the frequency of pathogen strains that harbor a mutation from avirulence to virulence (McDonald & Linde, 2002). The resistance breaking and on the contrary the resistance durability thus depend on this ability of the pathogen (or pest) to adapt and vary in response to the selection pressure (McDonald & Linde, 2002).

The main genes found out and characterized on different plant species for resistance to nematodes are listed in Table 1.1. All of them have been identified towards root-knot or cyst nematode species, i.e. sedentary endoparasitic nematodes, except *XiR1* for resistance to the ectoparasitic species *X. index*. These *R* genes differ by their resistance spectrum in particular: some *R* genes are specific to a single species, for example *Hs1^{pro-1}* and *Rmc2*, while others control a few (*Mi*, *RMia*, *RMja*, *Mur1* and *N*) or many (*Ma*) species (Table 1.1).

R genes finely mapped or cloned have a NB-LRR core structure and belong either to the TIR-NB-LRR (TNL) family (*Gro1-4*, *Ma*, *RMia* and *RMja*) or to the CC-NB-LRR (CNL) family (*Gpa2*, *Hero* and *Mi-1.2*). The gene *XiR1* (Xu et al., 2008) for resistance to *X. index* is a TNL gene located on the chromosome 19 of the grapevine genome (Table 1.1).

Table 1.1. Main plant genes identified for resistance to nematodes

Plant species	<i>R</i> gene	Nematode species	<i>R</i> gene class	References
Tomato <i>Solanum lycopersicum</i>	<i>Mi-1.2</i>	<i>M. arenaria</i> <i>M. incognita</i> , <i>M. javanica</i>	CNL	Ammati et al., 1985 Roberts & Thomason, 1989
Potato <i>Solanum tuberosum</i>	<i>Hero</i>	<i>G. rostochiensis</i>	CNL	Ganal et al., 1995
	<i>Gro1-4</i>	<i>G. rostochiensis</i>	TNL	Paal et al., 2004
	<i>Gpa2</i>	<i>G. pallida</i>	CNL	Van der Voort et al., 1997
<i>S. fendleri</i>	<i>Rmc2</i>	<i>M. chitwoodi</i>		Janssen et al., 1997
Pepper <i>Capsicum annum</i>	<i>Me1</i> , <i>Me3</i> , <i>Me7</i>	<i>M. incognita</i>		Djian-Caporalino et al., 2001

Cowpea	<i>Rk</i>	<i>M. arenaria</i>		Fery & Dukes, 1980
<i>Vigna unguiculata</i>		<i>M. incognita</i> , <i>M. javanica</i> <i>M. hapla</i>		
Sugar beet	<i>Hs1^{pro-1}</i>	<i>Heterodera</i> <i>schachtii</i>		Cai et al., 1997; Jung & Löptien, 1986
<i>Beta sativa</i>				
Myrobalan plum	<i>Ma</i>	<i>M. arenaria</i>	TNL	Esmenjaud et al., 1996
<i>Prunus cerasifera</i>		<i>M. incognita</i> <i>M. javanica</i> <i>M. floridensis</i>		Lecoals et al., 1997 Claverie et al., 2011
Peach	<i>RMia</i>	<i>M. arenaria</i>	TNL	Claverie et al., 2004
<i>P. persica</i>		<i>M. incognita</i>		Yamamoto & Hayashi, 2002 Duval et al., 2014
Almond	<i>RMja</i>	<i>M. arenaria</i>	TNL	Kochba & Spiegel-Roy, 1975
<i>P. dulcis</i>		<i>M. javanica</i>		Van Ghelder et al., 2010; 2018
Grapevine		<i>M. arenaria</i>		Reviewed in Saucet et al., 2016
<i>Vitis mustangensis</i> and <i>V. champinii</i>	<i>N</i> and <i>Mur1</i>	<i>M. incognita</i> <i>M. javanica</i>		
<i>V. arizonica</i>	<i>XiR1</i>	<i>X. index</i>	TNL	Xu et al., 2008

1.1.3.2. Biological and histological mechanisms

Highly specific nematode resistances can be divided into three major types based on their timing in the ontogeny of feeding structures and their characteristic cytological features (see Govere & Smart 2014 for a review).

The first type is a rapid hypersensitive response (HR) resulting in host cell death during the initiation of a feeding structure. It takes place early (in annuals, within 24 h of host invasion) and arrests the development of the nematode (Paulson & Webster, 1972). This phenomenon was observed in the interaction between RKNs and either the *Mi-1*-resistant tomato (Melillo et al., 2006) or the *Ma*- and *Me3*-mediated resistance from plum and pepper, respectively (Fig. 1.6) (Bleve-Zacheo et al., 1998; Khallouk et al., 2011).

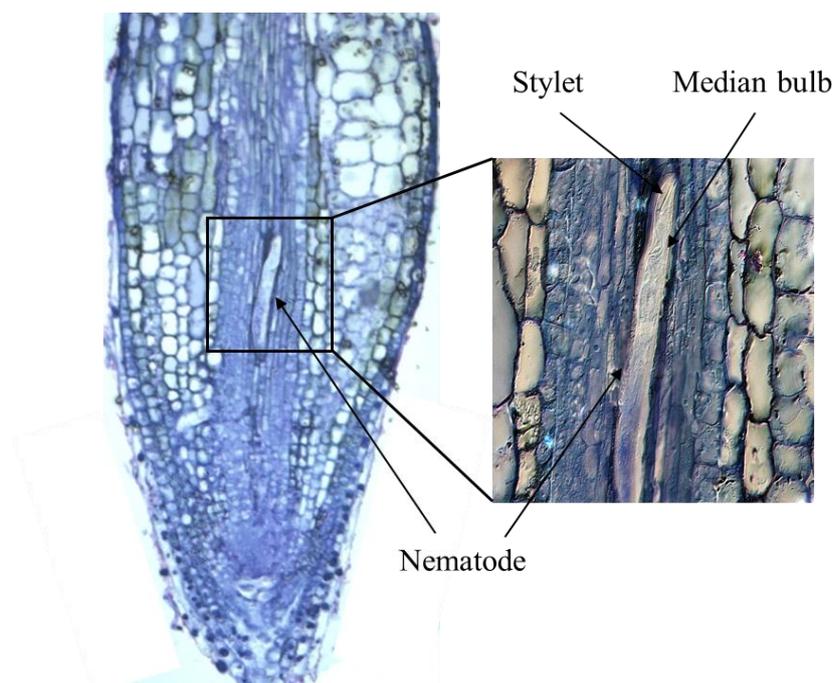


Figure 1.6. Incompatible interaction in a resistant plum accession carrying the *Ma* gene. The nematode at the vermiform 2nd-stage juvenile (J2) cannot induce the feeding structure (adapted from Khallouk et al., 2011)

The second type of resistance response allows for the initiation but restricts the expansion of nematode-induced feeding structures by the formation of a layer of necrotic cells around the feeding structure. This type was observed for the *H1* and *Hero* resistances to PCN in potato and tomato, respectively (Rice et al., 1985; Sobczak et al., 2005). A similar inhibition of the expansion of giant cells of RKNs by necrosis also favors the development of males (Kouassi et al., 2004).

The third resistance response type occurs later than the expansion of the feeding structures. This response causes insufficient food supply at this stage and blocks further growth and development of the females. This resistance response type can occur with or without HR (Das et al., 2008; Kandoth et al., 2011). For example, in potato plants with *Gpa2* resistance to PCN, a layer of dead cells between the syncytium and the vascular bundle was recorded 10 days after the invasion (Koropacka, 2010). Otherwise, the histological evolution of syncytia of the soybean cyst nematode *H. glycines*, in soybean genotypes harboring the *Rhg1* locus, is not characterized by a typical HR but by the collapse of the syncytia after nuclear and cytoplasmic disintegration (Kandoth et al., 2011).

1.1.3.3. Molecular mechanisms

At the molecular level, a two-layer detection system has evolved in plants for the purpose of pathogen recognition (Jones & Dangl, 2006; Dodds & Rathjen, 2010) (Fig. 1.7). (1) The basal defense is characterized by pattern recognition receptors (PRRs) localized at the cell

surface and recognizing pathogen or microbial-associated molecular patterns (PAMPs or MAMPs). PAMPs or MAMPs activate host defence responses (PAMP-triggered immunity or PTI) through a complex signalling cascade. (2) The second detection system focuses on effectors secreted by pathogens into the host. These effectors may interfere with PTI responses but are not recognized, leading to effector-triggered susceptibility (ETS). Plants may subsequently be able to recognize effectors by members of a family of intracellular proteins called nucleotide-binding leucine-rich repeat receptor proteins (NLRs). Activation of NLRs leads to a stronger and more intense defense response (effector-triggered immunity, ETI), which often culminates in a form of programmed cell death or hypersensitive response (HR). This phenomenon was well studied for *Mi-1* in tomato (Melillo et al., 2006), *Me3* in pepper (Bleve-Zacheo et al., 1998), *Ma* in plum (Khallouk et al., 2011) for resistance to *Meloidogyne* spp. and for *Hero A* in tomato (Sobczak et al., 2005) for resistance to *G. rostochiensis*,

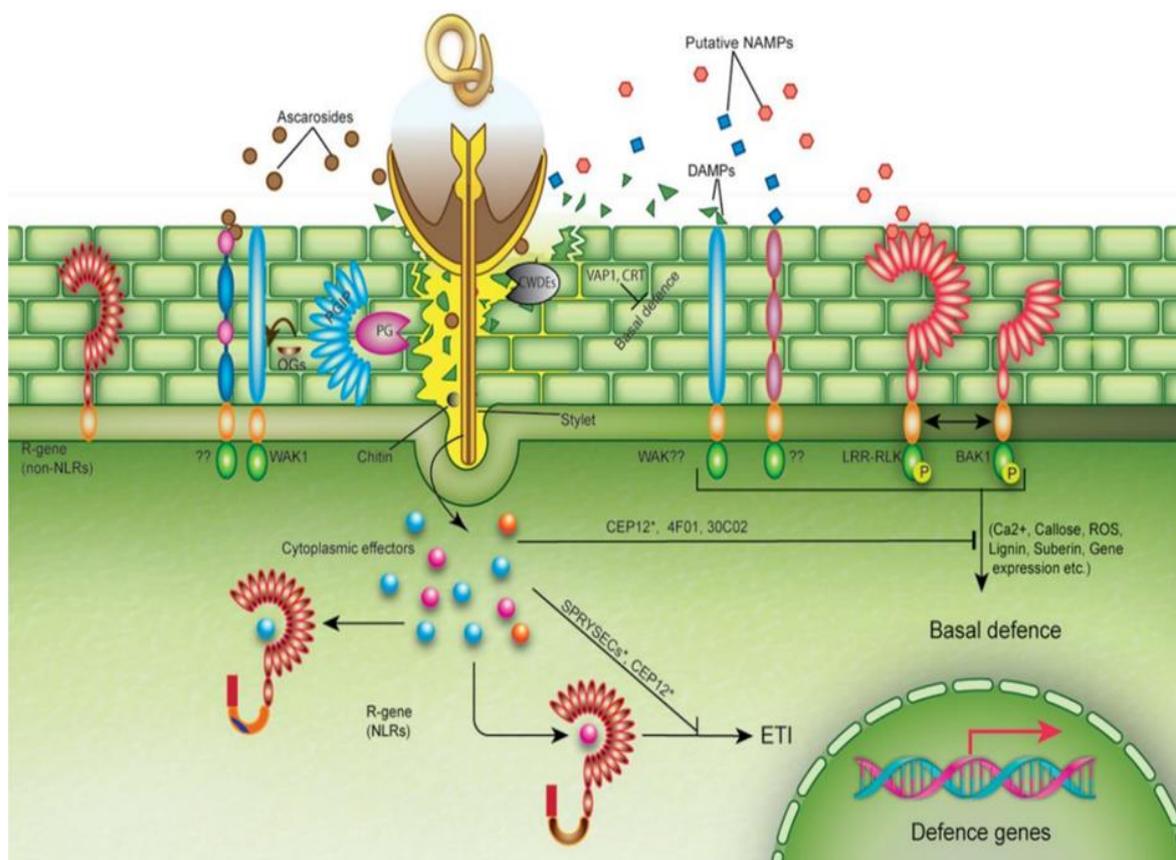


Figure 1.7. Resistance models during plant-nematode interaction. Nematodes may face two different plant defense layers: (1) basal defense such as WAK1, PG-inhibiting proteins (PGIPs), pattern-triggered immunity (PTI) including the production of reactive oxygen species (ROS), callose, and lignin. (2) *R* genes that recognize effectors either directly or indirectly and initiate effector-triggered immunity (ETI). *R* genes may be NLRs (nucleotide-binding leucine rich repeat) and non-NLRs type genes. (Holbein et al., 2016)

1.1.3.4. Interaction of resistance with plant hormones

Laboratory techniques have allowed the development of *in vitro* culture methods for intensive plant propagation in particular. Grapevine was among the first plants to be cultured *in vitro* and reproducible micropropagation techniques were first reported in the late 1960s (Galzy, 1969). *In vitro* culture begins with the excision of a small piece of plant tissue, freeing it from contaminating microorganisms and establishing it in sterile culture. *In vitro* propagation can be obtained by shoot development from nodal cuttings (Galzy, 1969), shoot apices (Harris & Stevenson, 1979; Goussard, 1981) and apical tips are commonly used to eliminate viruses and considered the best choice as explant type for general micropropagation purposes (Hu & Wang, 1983). Several hormones can be used during the *in vitro* culture and the primary classes are auxins and cytokinins. The most important synthetic auxins are 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 4-chlorophenoxyacetic acid (CPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2-naphthoxyacetic acid (NOA) and indole-3-acetic acid (IAA). Main cytokinins include 6-benzyladenine or benzylaminopurine (BAP), kinetin (Kin), 6-(2-isopentenyl) adenine (2iP), zeatin or zeatin riboside (ZR) and 3-(1,2,3,4-thiazol-5-yl)-1-phenylurea or thidiazuron (TDZ).

Several studies have reported the effect of hormones widely used in *in vitro* cultures on plant resistance. For example, it has been shown that exogenously supplied cytokinins shifted the response of resistant tomato plants (*S. lycopersicum*) carrying the *Mi* gene to *M. incognita* towards susceptibility (Dropkin et al., 1969) presumably because cytokinin activity was lower in resistant than in susceptible cultivars (Gheysen & Fenoll, 2002). This highlighted for the first time the cytokinin-mediated suppression of resistance and of the cognate hypersensitive response of tomato roots to the nematode. In the wild resistant tomato *S. sisymbriifolium*, resistance was lost in an *in vitro* medium supplemented with growth hormones (mix of auxin and cytokinin) while the sections of stems and leaves rooted in sand without hormones retained resistance. An additional test with a *Mi*-resistant tomato cultivar, regenerated from callus and thus using hormones, showed that plants retained resistance to *M. incognita* but not to *M. javanica* (Fassuliotis & Bhatt, 1982). In perennials, the influence of cytokinin concentration has also been recorded in the resistant peach rootstock Nemaguard grown in *in vitro* culture. At high concentration of cytokinin (BA) (2mg/L), the plant lost resistance to *M. incognita* by forming galls, even though the nematode failed to develop to maturity (incomplete life cycle) (Huettel & Hammerschlag, 1986). Previously, Kochba & Samish (1972) had shown that natural cytokinin activity measured in extracts from Nemaguard was significantly lower than in the susceptible control Baladi. This suggests that high cytokinin levels, either endogenous or applied, correlate with plant susceptibility to root-knot nematodes. Moreover, biologically active cytokinins have been shown to be produced by *Meloidogyne* spp. (Bird & Koltai, 2000). On *Prunus*, Esmenjaud et al. (1993) observed that

in vitro rooting of *P. cerasifera* in a medium supplemented with 0.5 ppm IBA did not influence the subsequent resistance of plants inoculated with *M. arenaria* 70 days after their acclimatization into a sandy substrate. Resistance of these plants was shown later to be conferred by the *Ma* gene (Esmenjaud et al., 1996). By contrast, treating resistant tomatoes (cv. Nematex) with a combination of NAA (0.05 ppm) and kinetin (1.07 ppm) altered them to susceptible by producing galls when inoculated with *M. incognita*, while this resistance alteration was not observed with NAA or kinetin applied separately (Sawhney & Webster, 1975).

1.1.3.5. Resistance durability

The aim of both breeder and grower is to increase as much as possible the resistance durability. Durability may be highly variable and efforts to obtain and use *R* factors may be neutralized after different time scales, i.e. from the end of a breeding program up-to over 130 years later such as in the case of the phylloxera aphid resistance of grape rootstocks (Pouget, 1990; Parlevliet, 2002). Theoretically, absolute durability is believed to not exist.

Definition of durable resistance

As reported above, the major limit to a wide use of genetic resistance against pests and pathogens is their ability to adapt to resistance factors. Johnson (1981; 1983) defined durable resistance as follows: pathogen resistance is sustainable if it remains effective when a variety is deployed in culture for a large time scale and under favorable conditions to the diseases (or pests). The Johnson's definition does not imply the genetic control of the resistance, its mechanism, or its degree of expression. To go beyond this definition, the reflection will move towards the evaluation of the *a priori* sustainability and resistance management. Therefore, integrated management aims to increase durability by combining management practices that will constrain the pest population to overcome multiple barriers and decrease the probability that a resistance-breaking genotype will appear. Resistance management is envisaged at two levels: i) the management of genes in planta, that is by introgression of genes or combinations of genes that will be *a priori* sustainable and ii) the management of genes in situ by the successive deployment of cultivars carrying different genes. Reflection can then commit to three scales: at the gene level, the individual level and the population level. One example of durable resistance is the *H1* gene for resistance to *G. rostochiensis* in potato (*Solanum tuberosum*). This gene was introduced and widely grown over 30 years but so far, no nematode virulent populations have been recorded (Castagnone-Sereno, 2002)

Mechanisms of durability

Parlevliet (1988) indicated that quantitative resistance (QR), closely linked to the notion of horizontal resistance according to Vanderplank, is on average more durable than major gene resistance, a resistance that is related to the vertical resistance. The durability of QR is commonly assumed to be due to the number of genes controlling the trait, though arguments to the contrary have been made (Vanderplank, 1978; 1982). Certainly, there are mechanisms other than gene number that could contribute to durability of QR. For example, selection coefficients against individual genes controlling QR will be smaller than those against major gene resistance. The degree of host genotype \times pathogen genotype specificity may be less for minor gene than major gene resistance. Host \times pathogen \times environment interaction could also play an important role for genes of minor effect (Kulkarni & Chopra, 1982). There have long been concerns that QR may select for pathogen virulence and/or aggressiveness (Mundt, 2014).

Pyramiding consists in stacking *R* genes into a single genotype or cultivar. Pyramiding *R* genes can be one solution to improve both durability and level of resistance (Nelson, 1972), because, in theory, the pathogen would need double or multiple mutations to overcome the resistance. Besides a quantitatively higher level of resistance, the additional value of pyramided *R* genes could be a prolonged durability and resistance against a broader spectrum of pathogen races. Therefore, pyramiding of several *R* genes in a single cultivar should be an effective strategy to control diseases (Stukenbrock & McDonald, 2008) or nematodes (Djian-Caporalino et al., 2014). Pyramiding *Me1* and *Me3* genes increased the resistance durability to *M. incognita* in pepper (*Capsicum annum*) (Djian-Caporalino et al., 2014), but this was not the case for *Bph1* and *Bph2* on rice against brown plant hopper in which resistance level of the pyramided line was equivalent to that of the *Bph1*-single gene (Sharma et al., 2004).

Resistance/virulence in plant-pathogen interaction

In an evolutionary sense, any resistance is ultimately transitory. In the gene-for-gene model (Flor, 1971), the interaction between a pest and its hosts is an endless race in the nature, where the pest attacks the host, which, in its turn, fights back with its own arsenal. This arms race is often supported by the fact that both host *R* genes and pest *Avr* genes undergo positive selection, which can lead to a coevolution between host resistance and pest virulence (Parlevliet, 1986; Kraaijeveld et al., 1998; Singh et al., 2018). Knowing how long a resistant plant (carrying *R* factors) is able to withstand the selection pressure of a pest is a major challenge. In the context of plant pathology, Shaner et al. (1992) defined that the 'virulent' pathogen is the genotype that can develop and reproduce on host plants carrying resistance genes. Hence, we used here 'virulence' in the standard plant pathology sense, meaning the nominal ability of the nematode to cause a susceptible response on a host plant carrying a given resistance gene. In plant-pathogen interactions, virulence has been identified in fungi, bacteria, viruses and nematodes (Castagnone-Sereno, 2002; Parlevliet, 2002; Lecoq et al., 2004).

The evolution of pathogens and of nematodes in particular may reduce the effectiveness of a resistance and makes it become shorter. According to McDonald & Linde (2002), pathogens with high evolutionary potential are more able to overcome a resistance than those with a lower evolutionary potential. These authors also predicted the risk of a pathogen by estimating the operating parameters of its populations such as the mode of reproduction, the population size, the gene and genotype flow (Fig. 1.8). Whereby, the highest risk belongs to the populations that have a mixed reproductive mode (both asexual and sexual reproductions), a big size, and whose subpopulations exchange many migrants (high gene flow). In such a population, recombination may make it possible to create new virulent individuals. During the asexual phase, the absence of recombination allows the survival and the multiplication of the virulent individuals and the establishment of a new virulent population in the environment.

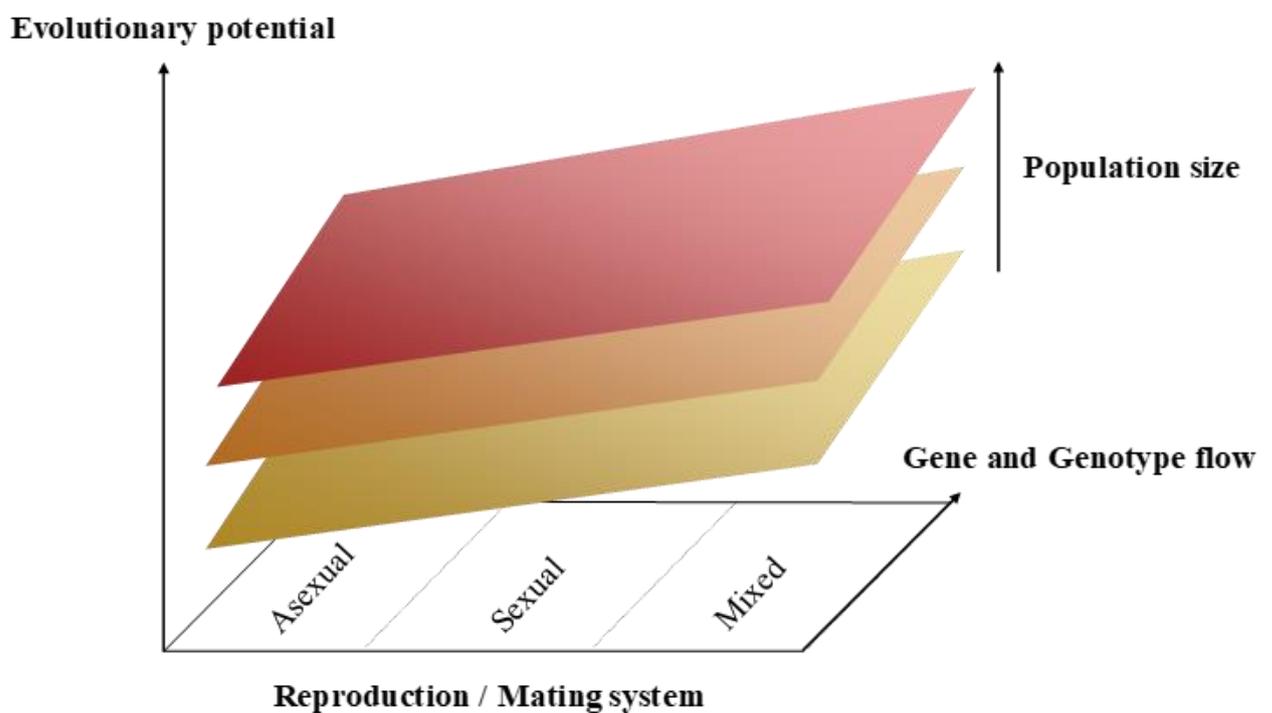


Figure 1.8. The impact of three factors (reproduction mode, gene and genotype flow, and population size) on the evolution of the pathogen or nematode (adapted from McDonald & Linde (2002) and Villate (2008)).

Nematode virulence

Virulence in sedentary endoparasitic nematodes

Virulence has been detected and studied in many sedentary nematodes. Cases of natural occurrence or laboratory selection of virulent *Meloidogyne* spp. populations have been described towards diverse *R* genes (Table 1.2).

For example, virulent *Meloidogyne* populations to the *Mi* tomato gene have been detected in many tomato growing areas in the world (Kaloshian et al., 1996; Eddaoudi et al., 1997; Ornat et al., 2001; Castagnone-Sereno et al., 2007; Devran & Söğüt, 2010). Reproduction of the nematodes was significantly higher on heterozygous than on homozygous tomato genotypes indicating a dosage effect of the *Mi* gene (Jacquet et al., 2005). In laboratory experiments from avirulent *M. incognita* populations in pepper, virulent variants could be selected toward the *Me3* gene but not towards the *Me1* gene (Castagnone-Sereno et al., 1996; 2001). In these RKNs, virulence to mitotic parthenogenetic nematodes is acquired progressively and mutated genes have been associated with the acquisition of resistance (Castagnone-Sereno, 2006; Castagnone-Sereno et al., 2007). As expected from their theoretical evolutionary potential (McDonald & Linde, 2002), virulence seems to be acquired quicker in the case of meiotic nematodes such as *M. hapla* and *M. chitwoodi* than in mitotic nematodes.

Table 1.2. Main resistance genes for which virulent populations of *Meloidogyne* spp. have been observed.

Plant species	Resistance gene	Nematode species	References
<i>Lycopersicon esculentum</i>	<i>Mi</i>	<i>M. incognita</i> , <i>M. javanica</i>	Castagnone-Sereno et al., 1996, 2007 Djian-Caporalino et al., 2011 Eddaoudi et al., 1997 Kaloshian et al., 1996
<i>Capsicum annum</i>	<i>Me1</i> , <i>Me3</i> , <i>Me7</i>	<i>M. incognita</i>	Castagnone-Sereno et al., 1996, 2007 Djian-Caporalino et al., 2011
<i>Vigna unguiculata</i>	<i>Rk</i>	<i>M. incognita</i>	Petrillo et al., 2006 Petrillo & Roberts, 2005a, 2005b Roberts et al., 1996
<i>Phaseolus vulgaris</i>	Single major gene	<i>M. hapla</i>	Chen & Roberts, 2003
<i>Solanum fendleri</i>	<i>Rmc2</i>	<i>M. chitwoodi</i>	Janssen et al., 1998

Studies on the durability of resistance to RKNs have also been conducted in the perennial *Prunus* species (Table 1.1) carrying the *Ma* plum gene for complete-spectrum resistance and the *RMia* peach gene for a more restricted spectrum. No breaking of resistance conferred by each of these genes has been observed after 4 years to *M. incognita*, while virulent populations to this RKN species could be obtained after 2 years from tomatoes carrying the *Mi* gene (Khallouk, 2013). In grapevine, resistance to *M. arenaria* and *M. incognita* from *V. champinii* in the Californian rootstocks Ramsey, Freedom and Harmony (carrying the *N* grapevine gene) has been overcome by certain populations of these two RKN species. New resistant rootstocks have been selected using *V. mustangensis* (gene *Mur1*) as the resistance source (see Saucet et al. 2016 for a review).

In cyst nematodes, the sexual reproduction may allow a rapid adaptation of the nematodes to the major *R* genes or *R* QTLs which they are faced to (Bird et al., 2015; Fournet et al., 2018)

Virulence in ectoparasitic nematodes

Contrary to sedentary endoparasitic nematodes in Solanaceous crops for which the adaptative phenomena underlying virulence to a specific *R* gene are now better understood, virulence has never been studied in migratory ectoparasitic nematodes. A single *R* gene (*XiR1*) has been evidenced to *X. index* but this gene has not yet been used in breeding and no data are available on its durability (Xu et al., 2008).

Virulence or adaptation are more difficult to study, given that these species remain in the soil while feeding from the plant and are less dependent on the plant state. At their larval or adult stages, dagger nematodes in general and *X. index* in particular have a higher natural potential to survive than sedentary endoparasitic nematodes. Indeed, these latter nematodes are extremely fragile when they have undergone the fixed endophytic part of their cycle. If they fail to complete it, they cannot survive while migratory ectoparasitic nematodes may remain alive, even though their feeding is unsuccessful. Consequently, even though it has been based on results obtained up-to 6 years, our study on the resistance durability of *X. index* developed further will be blurred by the fact that the nematode can survive for several years in the soil.

I.2. THE GRAPEVINE

In the Vitaceae family, the *Vitis* genus consists of ~ 60 interfertile species that exist almost exclusively in the Northern Hemisphere and out of which approx. 15 have been used in breeding programs (Table 1.3).

Table 1.3. Main *Vitis* species used in breeding programs (adapted from This et al., 2006)

Species	Used for breeding cultivars	Used for breeding rootstocks	Used for breeding interspecific hybrids
<i>Vitis vinifera</i>	+++++	+	++++
<i>V. aestivalis</i>	-	-	++
<i>V. amurensis</i>	+	-	++
<i>V. berlandieri</i>	+	+++	-
<i>V. candicans</i>	-	+	-
<i>V. caribaea</i>	-	-	+
<i>V. champinii</i>	+	+	-
<i>V. cinerea</i>	-	+	++
<i>V. cordifolia</i>	-	+	+
<i>V. labrusca</i>	+++	++	+++
<i>V. longii</i>	+	++	-
<i>V. riparia</i>	++	+++	+++
<i>V. rupestris</i>	++	+++	+++
<i>V. simpsonii</i>	-	+	-
<i>Muscadinia rotundifolia</i>	++	+	+

1.2.1. The *Vitis vinifera* grape

1.2.1.1. Botanical and genetic data

This species is the wine producer and the producer of table grapes and raisins. Currently, there are more than 10,000 varieties of *V. vinifera*, but only a few are of commercial significance for wine production (Sechrist, 2017). Cultivars of *V. vinifera* form the basis of the majority of wines produced around the world. All of the familiar wine varieties belong to *V. vinifera*, which is cultivated on every continent except Antarctica. Domesticated vines have hermaphrodite flowers.

The grapevine (*V. vinifera* L.) is diploid and has a genome size of 475–504.6 Mb (Thomas et al., 1993; Lodhi & Reisch, 1995; Jaillon et al., 2007; Velasco et al., 2007), consisting of 19 chromosomes. In 2006, Lowe & Walker released the first interspecific linkage map of rootstocks parents (*V. champinii* cv. Ramsey x *V. riparia* cv. Gloire de Montpellier). In 2007,

two high-quality draft genome sequences from the variety 'Pinot Noir' of *V. vinifera* were released (Jaillon et al., 2007; Velasco et al., 2007). The genotypes of grape varieties are highly heterozygous and nearly all modern cultivated varieties (cultivars) are hermaphroditic, self-fertile and out-cross easily. The International Grape Genome Program (IGGP), formed in 2001, promoted international collaboration and developed public resources for the grape research community (<http://www.vitaceae.org/>). At the time of this writing, a summary of data deposited at NCBI for *V. vinifera* is as follows: 355,541 nucleotide sequences, 446,674 ESTs, 229,272 GSS that when clustered produced a UniGene set of > 32,193 genes (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=29760&lvl=3&p=mapview&p=has_linkout&p=blast_url&p=genome_blast&lin=f&keep=1&srchmode=1&unlock).

1.2.1.2. Evolution and history

The grape has an ancient historical connection with the development of human culture. The grapevine (*V. vinifera*) might have grown wild in the Caucasus foothills of modern Georgia, Azerbaijan and Armenia 100,000 years ago. The domestication of grape seems linked to the discovery of wine. At first, people could not store the wine and consumed it only seasonally. The invention of pottery allowed year-round storage at least 9,000 years ago (Sechrist, 2017). To date, two forms (wild and cultivated) still co-exist in Eurasia and in North Africa: the cultivated form, *V. vinifera* subsp. *vinifera* (or *sativa*) and the wild form *V. vinifera* subsp. *sylvestris* (or *sylvestris*), sometimes referred to as a separate subspecies (See This et al., 2006 for a review). During domestication, the biology of grapes underwent several dramatic changes to ensure greater sugar content for better fermentation, greater yield and more regular production. Although wild grapes were probably present in many places in Europe during the Neolithic period, archaeological and historical evidence suggest that grapevine domestication occurred in the Near-East (McGovern et al., 1996; McGovern, 2003; Sechrist, 2017).

Historical studies (Zohary, 1996; This et al., 2006; Sechrist, 2017) completed by robust genetic markers (chloroplast DNA, microsatellites) (Arroyo-García et al., 2006; Bacilieri et al., 2013) allow to propose a hypothesis of the dissemination of grapevine from its native area (Fig. 1.9). That diffusion of viticulture from the Near East has occurred through two ways around the Mediterranean Basin: (1) a Northern way from Eastern to Western Europe by Hittite, Greek and Roman people in the Antiquity (Sechrist, 2017) and (2) a Southern way through Mesopotamia, Egypt and the Iberian Peninsula by Phoenicians in the Antiquity and

Arabs later. Domesticated grapevine finally reached the new world (North and South America) in 16-17th century by the Spanishs (Fig. 1.9).

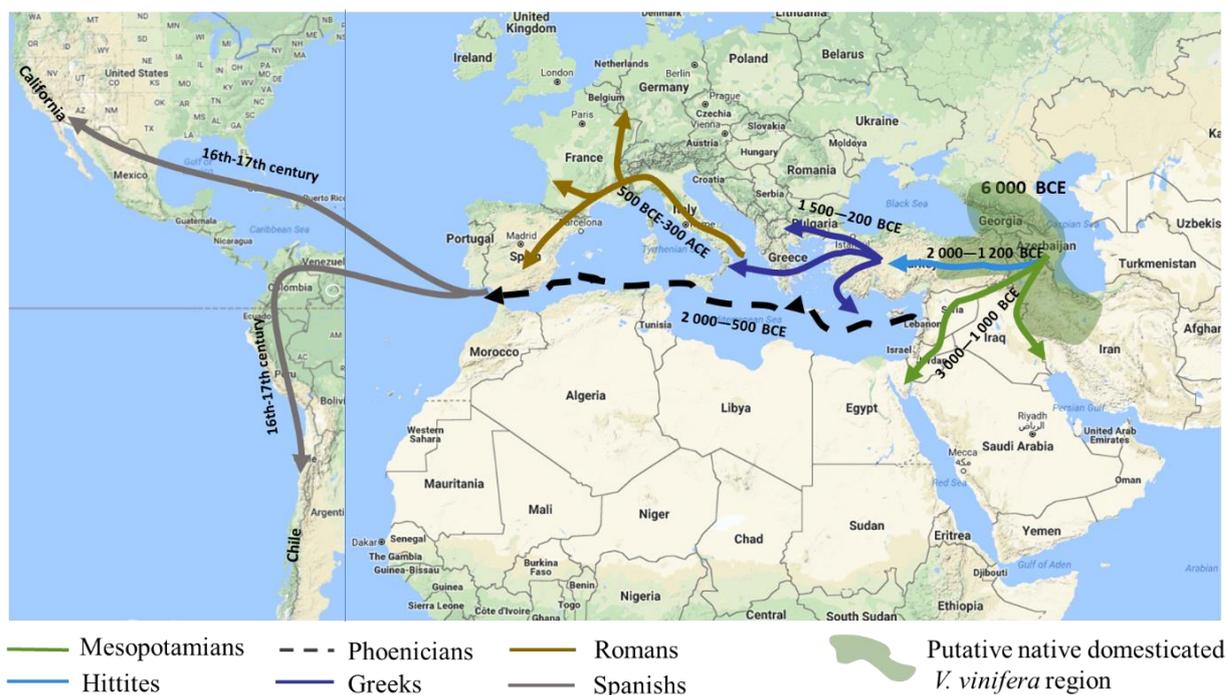


Figure 1.9. Dissemination of domesticated grapevine from its putative native area. The primary center of domestication is indicated in the Transcaucasian (dark green).

1.2.2. The muscadine grape

The muscadine grape (*Muscadinia rotundifolia* Michx.) is native to the southern United States and has been cultivated for over 400 years (Lane, 1997). In this thesis, the name “muscadine” will be used exclusively for *M. rotundifolia*. The muscadine grape differs from the familiar bunch grape (*V. labrusca*, *V. vinifera*) in several morphological characteristics, including that it has smaller clusters, its berry has a thick skin and a unique fruity aroma. *Muscadinia* grapes have 40 somatic chromosomes ($2n = 40$) and are characterized by fruits borne in many clusters with few berries per cluster. A long-standing goal of both *Vitis* and *M. rotundifolia* breeding programs has been the development of hybrids between these groups, combining fruit quality from *V. vinifera* with disease resistance and environmental adaptation of muscadines (Bouquet, 1980; Olmo, 1986; Goldy et al., 1988).

1.2.3. Grapevine rootstocks

Nowadays, more than 80% of the vineyards worldwide use grafted plants (Smith, 2004). Those are scions of *V. vinifera* grafted onto a rootstock that contains one or several desirable features like abiotic or biotic stress resistances.

1.2.3.1. Origin of rootstock use in viticulture

The root aphid phylloxera (*Daktulosphaira vitifolia* Fitch) was introduced in Europe (1858-1860) through the importation of infected rooted vines from North America (Pouget, 2015). The pest led to the complete destruction of the vineyards in southern France at the end of the nineteenth century and, within 20 years, phylloxera had killed virtually every vineyard in France. In 1869, Laliman – a winegrower in Bordeaux – was the first to suggest a genetic control of phylloxera pest by grafting the susceptible wine varieties onto the American phylloxera-resistant rootstock species (Pouget, 2015). Therefore, the introduction of several indigenous American non-*vinifera* *Vitis* species (Table 1.3) in Europe was urgently performed at that time to be used directly as phylloxera-resistant rootstocks or as source materials for the creation of resistant rootstocks. Some of them are still useful now as source material for resistance to grapevine fungi such as downy and powdery mildews. Because it had solved the problem of the phylloxera epidemics, grafting onto ‘American’ *Vitis* rootstocks became, from that period, an obligate practice in viticulture. It is common now in all regions where grapevine is the main crop, except in some isolated areas or regions that have not been contaminated by the aphid or that benefit from particular soil conditions (pure sand, winter flooding...).

1.2.3.2. Selection and creation of grapevine rootstocks

The majority of rootstocks used today are hybrids involving several of the three species *V. berlandieri*, *V. riparia* and *V. rupestris* (Galet, 1998). Because of the rootstock use, the cultivated grapevine is a combination of two genomes. The rootstock genotype represents the root system of the vine, i.e. the link between the edaphic environment and the plant. Rootstocks are responsible for water and mineral uptake and play an important role for storage. The grafting point defines the interface between shoot (above ground organs, i.e. the cultivar of *V. vinifera*) and root (below ground organs, i.e. the rootstock cultivar). From the first discovery of phylloxera-resistant rootstocks up to now, grafting sensitive *V. vinifera* varieties onto resistant rootstocks of North American origins has been the only viable long-term and sustainable solution for grapevine phylloxera management. Using resistant rootstocks are also a privileged way to select for grapevine resistance or adaptation to drought, diseases and pests (including nematodes) (Ollat et al., 2016). Muscadines exhibit good resistance traits to pests in particular, making them promising components of rootstocks in viticulture but they show poor performances to key abiotic features such as adaptation to dry and calcareous soils. Hybridization with appropriate *Vitis* species should allow in the

future to combine these abiotic features with resistances to pests and diseases. The rootstock resistance to *X. index*, notably in *Muscadinia* material, will be discussed in detail further in the paragraph '1.4.2' entitled 'The resistance against *X. index*'.

I.3. THE FANLEAF DEGENERATION OR 'COURT-NOUÉ' DISEASE

Currently, approximately 60 viruses belonging to different families have been reported on grapevines (*Vitis* spp.) (Martelli, 2014). Therefore, grapevine is the crop with the highest number of viral diseases. Among the grapevine viruses, *Grapevine fanleaf virus*, *Grapevine leafroll disease*, *Grapevine rugose wood disease* and *Grapevine fleck virus* are the four main disorders having a worldwide economic importance (Andret-Link et al., 2004; Naidu et al., 2015), and several others such as *Red blotch virus* or *Grapevine pinot gris virus* are considered as emerging (Maliogka et al., 2015; Basso et al., 2017).

I.3.1. The viruses involved in the 'Court-noué' disease

The fanleaf degeneration of grapevine or 'Court-noué' disease is due to several viruses that all belong to the *Nepovirus* group. Among them, GFLV, transmitted by *X. index*, is by far the most frequent. Another major agent of 'Court-noué' is *Arabis mosaic virus* (ArMV) transmitted by *X. diversicaudatum*. The other causal agents of fanleaf degeneration are mainly *Tomato black ring virus* (TBRV) transmitted by *L. attenuatus*, *Tobacco ringspot virus* (TRSV), *Grapevine chrome mosaic virus* (GCMV) and *Tomato ringspot virus* (ToRSV) (Sanfaçon et al., 2009) (Table 1.4). In 1970, Cohn & Nitzany reported that *X. italiae* is another vector of GFLV but this result has never been confirmed (Martelli & Taylor 1990; Taylor & Brown 1997). Thus, it is unlikely that *X. italiae* acts as a specific vector of GFLV (Brown et al., 1995). Consequently, *X. index* is admitted as the unique vector for GFLV.

In the thesis, we will simplify by considering GFLV as the causal agent of 'Court-noué' or grapevine fanleaf degeneration and *X. index* as its sole vector nematode.

Table 1.4. *Longidoridae* nematode vectors and their associated viruses (adapted from Andret-Link et al., 2017)

Nematode vector species		Virus names	Acronym	Main geographical location
<i>Xiphinema</i>	<i>americanum</i>	<i>Peach rosette mosaic virus</i>	PRMV	Worldwide
	'sensu lato'	<i>Tobacco ringspot virus</i>	TRSV	
		<i>Tomato ringspot virus</i>	ToRSV	
	<i>americanum</i>	<i>Tobacco ringspot virus</i>	TRSV	North America
	'sensu stricto'	<i>Tomato ringspot virus</i>	ToRSV	
	<i>bricolensis</i>	<i>Tomato ringspot virus</i>	ToRSV	North America
	<i>californicum</i>	<i>Tobacco ringspot virus</i>	TRSV	North and South America
		<i>Tomato ringspot virus</i>	ToRSV	
	<i>diversicaudatum</i>	<i>Arabidopsis mosaic virus</i>	ArMV	Europe
		<i>Strawberry latent ringspot virus</i>	SLRSV	
<i>index</i>	<i>Grapevine fanleaf virus</i>	GFLV	Worldwide	
<i>rivesi</i>	<i>Tobacco ringspot virus</i>	TRSV	North and South America	
	<i>Tomato ringspot virus</i>	ToRSV		
	<i>Tomato ringspot virus</i>	ToRSV		
<i>Longidorus</i>	<i>apulus</i>	<i>Artichoke Italian latent virus</i>	AILV	Italy
	<i>attenuatus</i>	<i>Tomato black ring virus</i>	TBRV	Europe
	<i>diadecturus</i>	<i>Peach rosette mosaic virus</i>	PRMV	North America
	<i>elongatus</i>	<i>Tomato black ring virus</i>	TBRV	Europe
		<i>Raspberry ringspot virus</i>	RpRSV	
	<i>macrosoma</i>	<i>Raspberry ringspot virus</i>	RpRSV	Europe
<i>Paralongidorus</i>	<i>maximus</i>	<i>Raspberry ringspot virus</i>	RpRSV	Europe

The host range of GFLV includes several species of *Chenopodium* and *Nicotiana* (Dias, 1963). Although *Cynodon dactylon* (Bermuda grass) was reported to be a host (Izadpanah et al., 2003), the primary host of GFLV in the agroecosystem is grapevine (Andret-Link et al., 2004).

1.3.2. The *Grapevine fanleaf virus*

GFLV is a plant pathogenic virus that belongs to the genus *Nepovirus* of the family Secoviridae. It is transmitted from grapevine to grapevine by the ectoparasitic nematode *X. index* (Hewitt et al., 1958).

1.3.2.1. Symptoms and impact

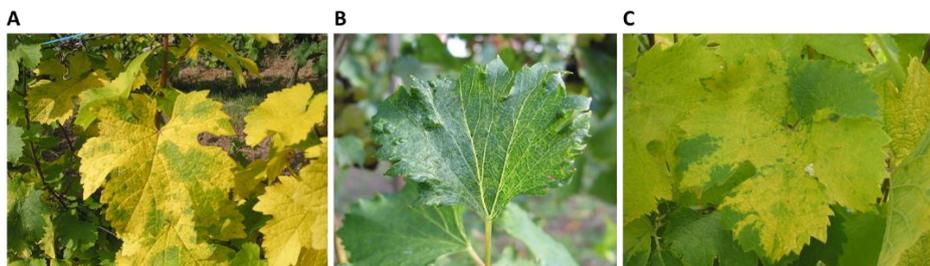


Figure 1.10. GFLV symptoms on grapevine. A & C: Yellowing symptom. B: Leaf malformation

GFLV is responsible for fanleaf degeneration called “court-noué”, the most severe viral disease of grapevines. This is a worldwide distributed virus, reported in North and South America, Europe (France, Italy, Spain...), Australia, etc. GFLV causes diverse symptoms and the most frequent are leaf yellowing, mottling and vein clearing, leaf distortion, fanleaf shape (the origin of the name ‘*Grapevine fanleaf virus*’), short internodes and stunted aspect of the plants (origin of the French name ‘Court-noué’) (Fig. 1.10). All of them induce lower fruit quality (with grape ‘coulure’ and ‘millerandage’ symptoms on bunches) and lower yields (Andret-Link et al., 2004). To the French grape and wine industries, “Court-noué” causes annual losses estimated of ~1 billion (Andret-Link & Fuchs, 2005). Once infected, the grapevine will decrease its productive life to 15-20 years instead of 30-40 years or longer with a healthy plant. That is an enormous and highly worrying problem in premium vineyards. Once grapevine is infected with the virus, along with the presence of *X. index*, dissemination of the vector nematode with the soil will help to spread it at a wide scale and will increase the impact of the disease. A survey conducted in 2004 by ONIVINS (Office National Interprofessionnel des Vins) indicated that only 30% of French vineyards were uninfected by ‘Court-noué’ viruses (Fig. 1.11).

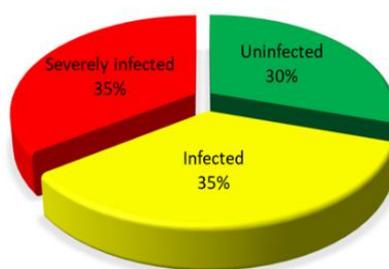


Figure 1.11. Infection of French vineyards in a survey conducted by ONIVINS in France (2004). 70% of French vineyards were infected (of which 35% were severely infected) and only 30% were free of virus.

1.3.2.2. The GFLV structure

GFLV particles are polyhedral with a diameter of 28 nm and consist of three serologically indistinguishable density components called top (T), middle (M), and bottom (B) (Quacquarelli et al., 1976; Brown et al., 1995; Mayo & Robinson, 1996). T component particles are empty shells, M component particles contain RNA2, and B component particles contain both RNA species (Quacquarelli et al., 1976). The GFLV genome consists of two single strands positive sense RNA1 and RNA2, with a molecular weight of 2.4×10^6 for RNA1 and 1.4×10^6 for RNA2. The two genomic RNAs carry a small covalently linked viral protein (VPg) at their 5' extremity and a poly(A) stretch at their 3' end (Pinck et al., 1988). RNA1 is 7,342 nucleotide (nt) long and contains a single open reading frame of 6,855 nt, extending from nt 243 to 7097 (Ritzenthaler et al., 1991), coding for the 1A, 1B^{Hel} (helicase), 1C^{VPg}, 1D^{Pro} (protease) and 1E^{Pol} (RNA-dependent RNA polymerase) proteins. The RNA2 is 3,774 nt long with a single open reading frame of 3,330 nt, extending from nt 233 to 3562 (Serghini et al., 1990) coding for the 2A^{HP} (homing protein), 2B^{MP} (movement protein) and 2C^{CP} (coat protein) proteins. The CP is composed of three domains called C, B, and A (Schellenberger et al., 2011) (Fig. 1.12). Infectivity requires both RNA1 and RNA2 (Quacquarelli et al., 1976).

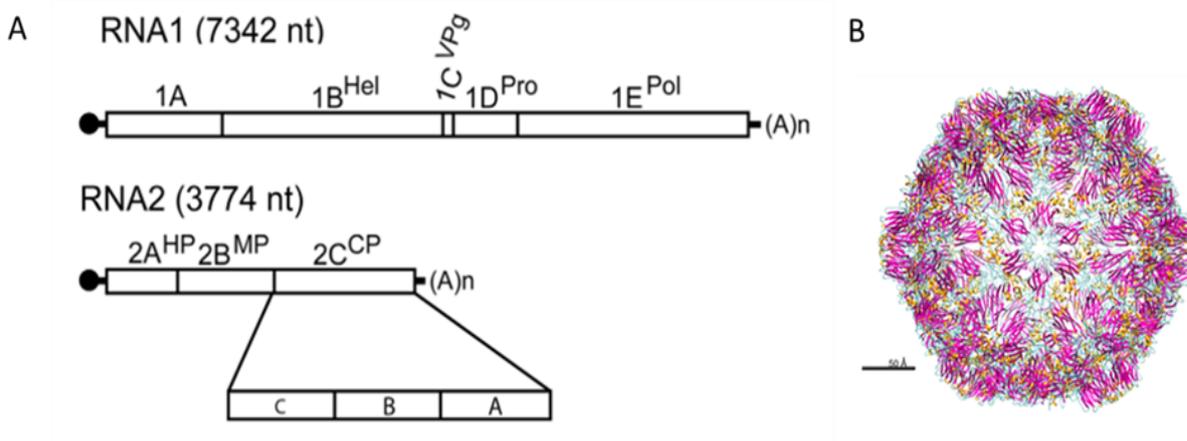


Figure 1.12. GFLV structure. **(A)** GFLV genetic features. The 5' and 3' untranslated regions are denoted by single lines and the VPg is represented by a black circle. Polyproteins encoded by RNA1 and RNA2 are cleaved in five (1A–1E) and three (2A–2C) final maturation products (open boxes), respectively. 1B^{Hel} (helicase); 1C^{VPg} (viral protein genome-linked); 1D^{Pro} (protease); 1E^{Pol} (RNA-dependent RNA polymerase); 2A^{HP} (homing protein); 2B^{MP} (movement protein) and 2C^{CP} (coat protein). Finally, the CP is composed of three domains from left to right called C, B, and A, respectively. **(B)** Crystal structure of GFLV capsid (adapted from Schellenberger et al., 2011).

1.3.2.3. Control of the virus

As there is no curative control of *Nepovirus*-infected grapevines in vineyard situations, the best way to control GFLV disease in a given area is to keep it out of this area through a system of quarantine, inspections and certifications. Once a grapevine plant is infected by the virus, it will be systematically disseminated into that plant (cell-to-cell movement) and it will become impossible to eliminate. Nevertheless, when the vector is not present in this area, prophylaxis using virus-free plant material is sufficient to solve the problem at the next generation. Hence, GFLV infection can be combatted by (1) using GFLV-free plants or propagation materials when the vector is not present in the grapevine field, (2) performing an effective management of *X. index* when the vector is present in order to slowdown the viral dissemination. Hereafter, we will focus on the first point about the virus eradication by summarizing i) the methods that allow the sanitation of virus-infected plant material, ii) the subsequent diffusion of this virus-free plant material through regulatory production schemes, iii) the natural resistance to GFLV in grapevine, and iv) the research approaches based on genetic engineering that may prevent in the future the virus multiplication in the plant.

Sanitation of GFLV-infected clones

GFLV-infected grapevine material, and high-value clonal accessions in particular, has been and can still be freed of viruses by heat therapy and/or meristem culture (Torres-Viñals et al., 2004; Gambino et al., 2009). Somatic embryogenesis has also been used to efficiently eliminate several phloem-limited viruses from grapevine material (Goussard et al., 1991). The success rate is close to 100% with this method without using heat therapy (Gambino et al., 2009).

Multiplication of virus-free plant material

At the national scale, organizations have been created to evaluate the plant material, eradicate the virus if needed, and then multiply or regulate the multiplication of virus-free plant material. Examples of such organizations are the National Grape Clean Plant Network (NCPN) in the US and the Canadian Plant Protection Export Certification Program (PPECP) for grapevine nursery stocks in Canada. In the same way in France, ONIVINS, established in 1983, oversees the entire wood and vine-seed industry and, as such, controls and certifies the propagating material of the vine. Below is a simplified flowchart for the sanitary selection of clones in France (revised in 2013) (Fig. 1.13). The aim is to get healthy clones from the infected initial grapevine clones of interest: plant material undergoes several steps with regular sanitary controls using appropriate molecular diagnostics (ELISA, PCR) and visual observations.

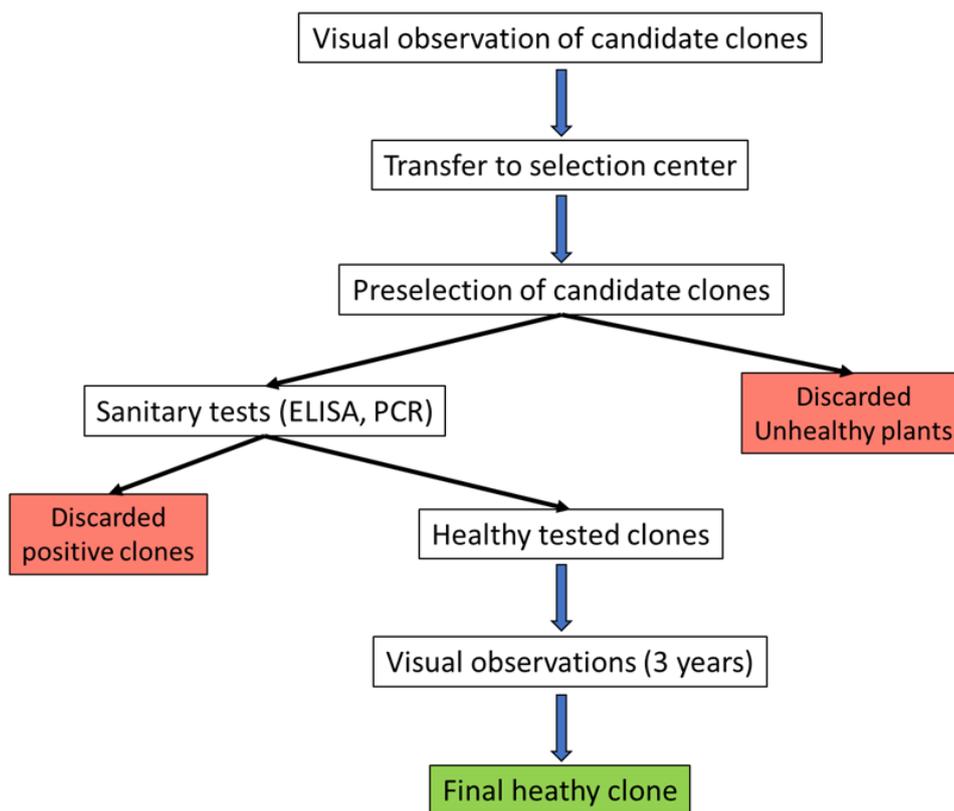


Figure 1.13. Technical regulatory scheme leading to the sanitation and approval of grapevine clones in France (from documents of the Official Directive 2005 for the French technical regulation of vine clones (2005/42/EC) modified in 2013).

Natural resistance to GFLV in grapevine

In California, several wild clonal accessions of *V. vinifera*, originating from Iran and Afghanistan, have been described as naturally resistant to GFLV (Walker & Meredith, 1990; Walker et al., 1985) but the results have not been confirmed and this research direction has been abandoned. In a complementary approach, the search for systematic detection of natural resistance to GFLV in *Vitis* material, conducted at INRA Colmar in France (Lahogue & Boulard, 1996) has been unsuccessful. Muscadines (*M. rotundifolia*) are hosts for GFLV even though this species and its hybrids with *Vitis* materials appear non or weakly affected by the virus (Walker et al., 1994a; Walker et al., 1994b) (A. Bouquet, pers. com.). Consequently despite the high diversity of *Vitis* and *Muscadinia* species (Myles et al., 2011; Reisch et al., 2012), over sixty years of GFLV resistance screening have not conclusively produced proven natural GFLV resistance in any grapevine genotype (Oliver & Fuchs, 2011).

Research approaches for virus control based on genetic engineering

Genetic engineering is an attractive way to develop GFLV-resistant rootstocks, especially since specific virus resistance traits can be inserted directly into desirable elite material (Fuchs, 2003). A highly-delayed infection has been reported in transgenic *Nicotiana*

benthamiana expressing the CP gene of GFLV (Bardonnnet et al., 1994). From that time many studies reported the obtention of transgenic grapevine lines expressing a GFLV-derived gene, but only a few of them showed the efficiency of the viral resistance induced (Jelly et al., 2012; Hemmer et al., 2017). Another way was using RNA silencing, a potent immune defense against viruses (Jardak-Jamoussi et al., 2009; Winterhagen et al., 2009; Gambino et al., 2010) or microRNAs (miRNAs). This latter approach is based on the use of miRNAs and their tailored modification for virus resistance. Modified miRNAs are referred to as artificial miRNAs (amiRNAs). Jelly et al. (2012) developed two artificial microRNAs (pre-amiRNAs) targeting the coat protein (CP) gene of GFLV for RNA silencing in grapevine somatic embryos. Very recently, Hemmer et al. (2017) used the nanobodies (Nb 23) obtained from camelid after 6 weekly injections of GFLV. The transgenic grapevine did not show any symptoms of GFLV or did not reveal the GFLV presence by ELISA tests at 45 days post-acclimatization. These data are very promising since they suggest that such transgenic grapevines are likely to be of practical interest for the control of GFLV.

However, only a few reports address the efficiency of these constructs to induce viral resistance in field conditions. Resistance to GFLV in transgenic rootstocks expressing the GFLV CP gene has been reported after a three-year trial in a naturally infected vineyard in France (Vigne et al., 2004). Nevertheless, until now and because of the non or at least difficult acceptance of GMOs by the civil society, there has not been any transgenic grapevine or rootstock grown at the field scale in French and European vineyards.

I.4. THE VECTOR NEMATODE *XIPHINEMA INDEX*

I.4.1. General information

I.4.1.1. Systematics of X. index and close grapevine Xiphinema species

Xiphinema index belongs to the order Dorylaimida and the family Longidoridae. The genus *Xiphinema* was first described by Thorne (1939) and *X. index* was first identified and described by Thorne & Allen (1951) (Fig. 1.18).

Systematics of Longidoridae species used to rely on morphological and morphometrical features. There were progressively completed by molecular sequences. Most often both the ribosomal sequences such as ITS1 (Vrain et al., 1992; Cherry et al., 1997), 28S (D2-D3 fragment) (Nunn, 1992) and 18S (SSU fragment) (Holterman et al., 2006), or the mitochondrial sequences such as the gene CO1 (He et al., 2005) were used. Using the 28S (D2-D3 fragment) sequence, Tzortzakakis et al. (2014) illustrated the phylogenetic relationships within the *Xiphinema* species (Fig. 1.14). The phylogenetic tree obtained allows to separate, with a high bootstrap support, most species and shows that both virus vectors *X. index* and *X.*

diversicaudatum are closely located while non vector species *X. vuittenezi* and *X. italiae* are distant and relatively grouped in the basal part of the dendrogram.

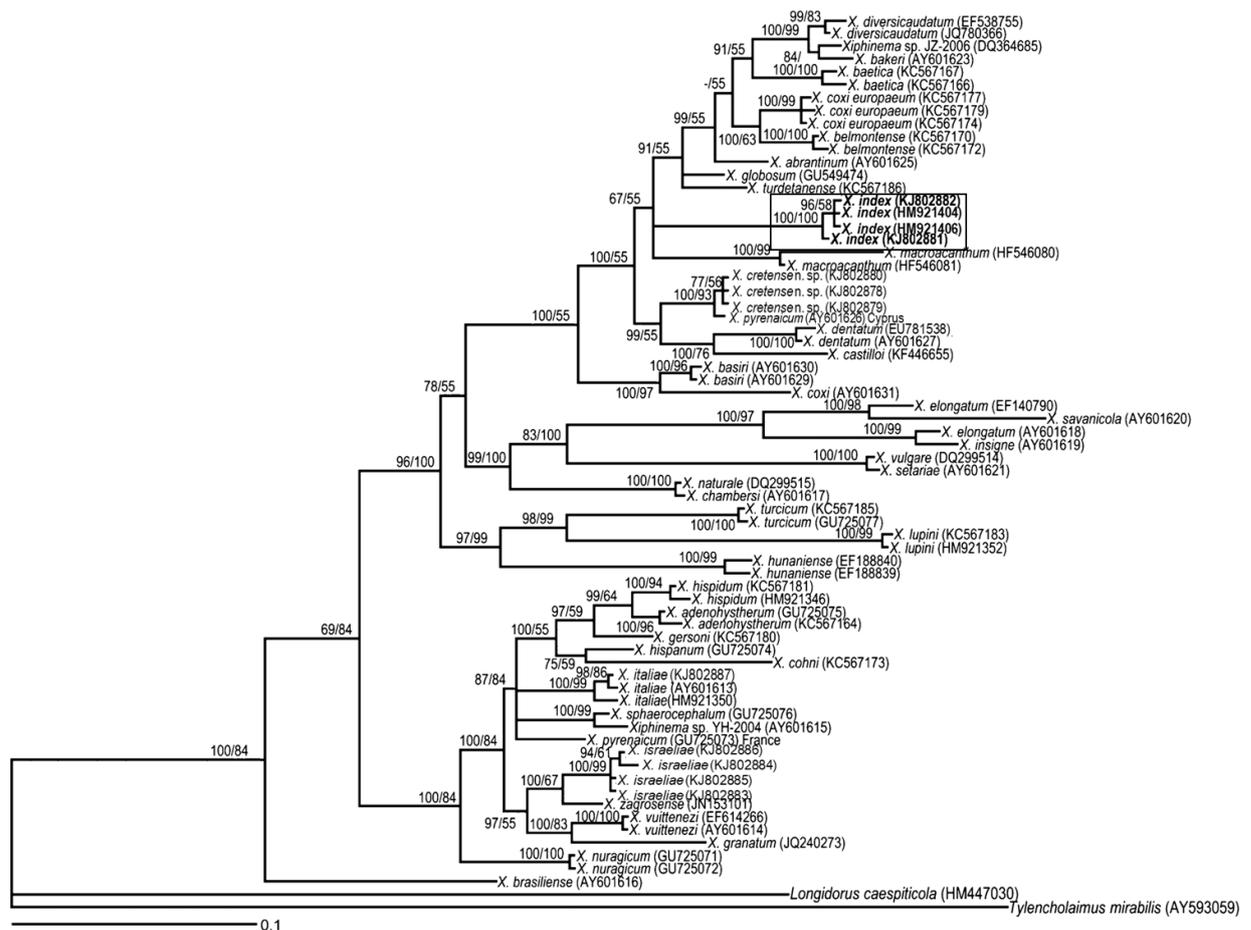


Figure 1.14. Phylogenetic relationships between *Xiphinema* spp. based on D2-D3 region of 28S rDNA. The nematode species *L. caespiticola* (Longidoridae) and *Tylencholaimus mirabilis* (Dorylaimidae) were used as outgroups (adapted from Tzortzakakis et al., 2014)

1.4.1.2. Morphology and anatomy

The *X. index* adult length ranges between 2.5 and 3.5 mm with a long protrusible stylet (190-215 μm) which allows it to reach the vascular tissue of plants (Fig. 1.18B). The stylet is composed of three characteristic basal flanges in its posterior end. The odontostyle is lined with a cuticle and alongside the odontophore and the esophagus serve as a surface for monolayer retention of GFLV particles (Fig. 1.23). Females have a vulva slit about half body-width long usually located at 38-40 percent of body length and two ovaries (Fig. 1.16 B). The male, with a similar body length, is rare and has paired spicules close to the anus (Thorne & Allen, 1951).

1.4.1.3. Geographic distribution

Xiphinema index is present in all major grape-growing regions in Europe, Asia, North and South America, Africa and Oceania (CABI source, Fig. 1.15). Its frequency is generally high in vineyards with a long history of grape cultivation. The nematode has most often been found associated with GFLV transmission to grapevine. *Xiphinema index* probably originated in the Middle East and was later introduced into different Mediterranean areas (Brown & Taylor, 1987; Villate, 2008).

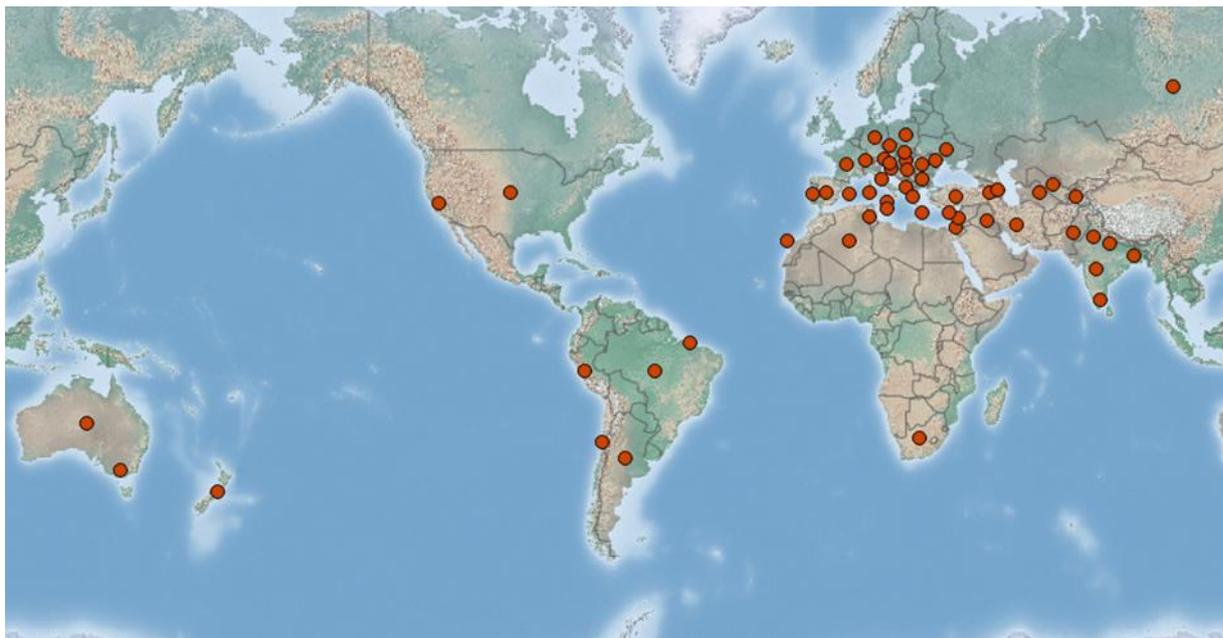


Figure 1.15. Distribution map of *X. index* (CABI, www.cabi.org/isc/datasheet/57032)

1.4.1.4. Identification

Besides *X. index*, several Longidorids species may be present in grapevine fields. These are mainly *X. diversicaudatum*, *X. vuittenezi*, *X. italiae* and *X. pachtaicum*. Some of them can be found in a same soil sample, particularly in European vineyards, and most often at low densities (Esmenjaud et al., 1992). Therefore, the species identification is the first important step before any control strategy.

Morphological and morphometrical identification

Species identification in *Xiphinema* used to be based mainly on classical diagnostic features. A combination of about 20 morphometric and morphological features has been established to elaborate dichotomous or polytomous keys for the identification of different nematodes species. The main diagnostic traits are body length, structure and size of the stylet, structure and position of the guide ring, shape and size of the lips, amphidials, and position

and size of the pharyngeal gland nuclei. Additional features at the species level are the development and structure of the female reproductive system, tail shape in all developmental stages, and presence or absence of males. Several keys that relied only on the observation of these features have been constructed. The most reliable and complete has been published in 1975 by Luc & Dalmaso and is usable under the form of a lattice, which is easier to handle than classical dichotomous keys. It does not consider the *Xiphinema* species from the so-called American group that contains many small-sized species of the American continent out of which some are virus vectors (*X. americanum sl*, *X. americanum ss*, *X. bricolensis*, *X. californicum*) (Table 1.4).

However, the high degree of intraspecific morphometric variability can lead to considerable overlap of many characteristics among *Xiphinema* species and increase the risk of species misidentification. Main morphological and anatomical features of the very close species *X. index* and *X. diversicaudatum* are reported in Fig. 1.16 as an example of similarity between species. Consequently, accurate complementary identification tools based on molecular sequences are useful.

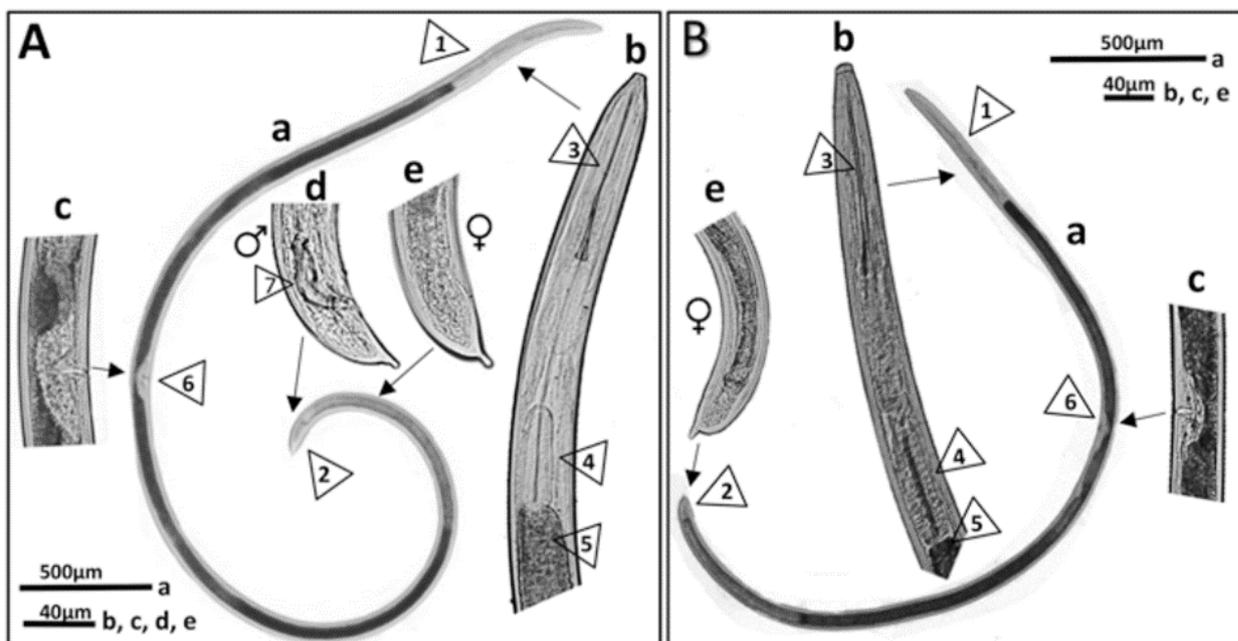


Figure 1.16. Morphological and anatomical features of the dagger nematodes *X. diversicaudatum* (A) and *X. index* (B). (a) A full body length with the head (arrow 1) and the tail (arrow 2); (b) head region showing a full stylet (arrow 3) and the esophageal bulb (arrow 4) followed by the intestine (arrow 5); (c) lateral view of the vulva (arrow 6); (d) lateral view of a male tail region with paired spicules (arrow 7); (e) lateral view of the female tail. Scale bars are indicated for each element (Andret-Link et al., 2017)

Molecular identification

Because of the poor reliability of the morphological and morphometrical traits, species identification has been efficiently completed by molecular markers. Recent description of new species use both the visually observable or measurable characters and diverse molecular sequences in order to propose a polyphasic identification (Gutiérrez-Gutiérrez et al., 2010; 2011)

In 2003, Wang et al. developed ITS1 ribosomal DNA sequences to design a set of SCAR markers for the identification of a few species that might be sympatric with *X. index* in grapevine: the vector species *X. diversicaudatum* and the non-vector species *X. vuittenezi*, and *X. italiae*. These markers are sensitive enough to identify a single nematode, regardless of its development stage (larvae or adult), within a nematode community in vineyard soils or within a mixed population consisting of non-vector and vector nematodes (Fig. 1.17). Nevertheless, because such SCARs can only distinguish the species that have been considered together for their primer design, their use cannot be universal. A much higher resolution identification can reasonably be obtained using sequences of genes or targeted fragments such as the ribosomal or mitochondrial DNAs. Thus, recently, numerous species from *Longidorus* and *Xiphinema* were molecularly characterized with such DNA as tools for species identification (Gutiérrez-Gutiérrez et al., 2011; Archidona-Yuste et al., 2016; Lazarova et al., 2016). Blasting on NCBI, the sequences obtained from appropriate primers (Table 1.5) showed that *X. index*, *X. diversicaudatum* and the non-vector species *X. vuittenezi*, *X. italiae* and *X. pyrenaicum* can be unambiguously distinguished from others and between each other. They constitute the basis of a universal barcoding adapted to grapevine nematode species (V.C. Nguyen and D. Esmenjaud, 2017, unpublished data from the INRA *Lycovitis* SMaCH project 2015-2017) (Table 1.5). Palomares-Rius et al. (2017) also illustrated that mitochondrial and ribosomal markers could be used as barcoding markers, except for some species from the *Xiphinema americanum* group. Another powerful identification method was developed recently by using real-time PCR for the specific detection of *X. index*, *X. diversicaudatum*, *X. vuittenezi*, and *X. italiae*. This sensitive and reliable technique might be useful to evaluate in the future the population densities in controlled experiments and putatively in vineyard soils (Van Ghelder et al., 2015)

At the intraspecific level, Villate (2008) proposed three sets of mitochondrial gene markers (*CytB*, *ND4* and *CO1*) to separate several *X. index* geographic populations with the DNA extracted from single individuals of each of them.

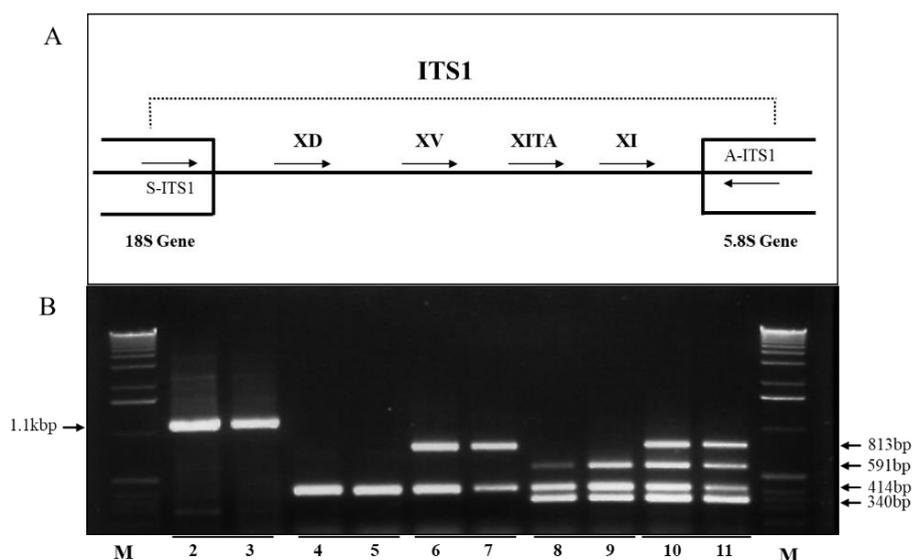


Figure 1.17. *Xiphinema* spp. identification based on the ITS1 ribosomal fragment located between the conserved sequences of the 18S and 5.8S genes. A: The ITS1 region and location of the 6 markers XD (*X. diversicaudatum*), XV (*X. vuittenezi*), XITA (*X. italiae*), XI (*X. index*), and control primers (S-ITS1 for sense (located on 18S gene) and A-ITS1 for antisense (located on 5.8S gene) to amplify ITS1 fragment). B: Electrophoresis of the amplification products from DNA isolated from *X. diversicaudatum*, *X. vuittenezi*, *X. italiae* and *X. index* in a multiplex test. DNAs were obtained from single individuals. Lanes 1 and 12, M (DNA ladder); lanes 2 and 3, 1.1-kbp control ITS1 fragment from *X. index*; lanes 4 and 5, *X. italiae*; lanes 6 and 7, *X. diversicaudatum* + *X. italiae*; lanes 8 and 9, *X. vuittenezi* + *X. italiae* + *X. index*; lanes 10 and 11, *X. diversicaudatum*+ *X. vuittenezi* + *X. italiae* + *X. index* (adapted from Wang et al., 2003).

Table 1.5. Markers for barcoding identification of the grapevine nematode species *X. index*, *X. diversicaudatum*, *X. italiae*, *X. pachtaicum*, *X. vuittenezi*, *P. vulnus*, and *L. attenuates* (V.C. Nguyen and D. Esmenjaud, 2017: unpublished data from the INRA *Lycovitis* SMaCH project - 2015-2017)

Sequence or gene	Primer	Primer sequence (5'-3')	Reference
<i>ITS1</i>	ITS1-18S-F	TTGATTACGTCCTGCCCTTT	Vrain et al., 1992
	ITS1-rDNA1-R	ACGAGCCGAGTGATCCACCG	Cherry et al., (1997)
<i>18S</i> (SSU)	998-F	CTCAAAGATTAAGCCATGC	Holterman et al., 2006
	1912-R	TTTACGGTCAGAACTAGGG	
	1813-F	CTGCGTGAGAGGTGAAAT	
	2646-R	GCTACCTTGTTACGACTTTT	
<i>28S</i> (D2-D3)	D2A-F	ACAAGTACCGTGAGGGAAAGTTG	Nunn, 1992
	D3B-R	TCGGAAGGAACCAGCTACTA	
<i>CO1</i>	COI-F	GATTTTTTGGKCATCCWGARG	He et al., 2005
	COI-R	CWACATAATAAGTATCATG	

1.4.1.5. Biology and ecology

Life cycle and reproduction mode

Xiphinema index primarily feeds on the root tips of grape rootstocks (*Vitis* spp.). The reproduction of *X. index* is mainly meiotic parthenogenetic (Fig. 1.19 B) and a single juvenile is capable of establishing a population (Dalmasso & Younes, 1969). Nevertheless, the occurrence of rare sexual reproduction events has been shown in the field (Villate et al., 2010) (Fig. 1.19 A).

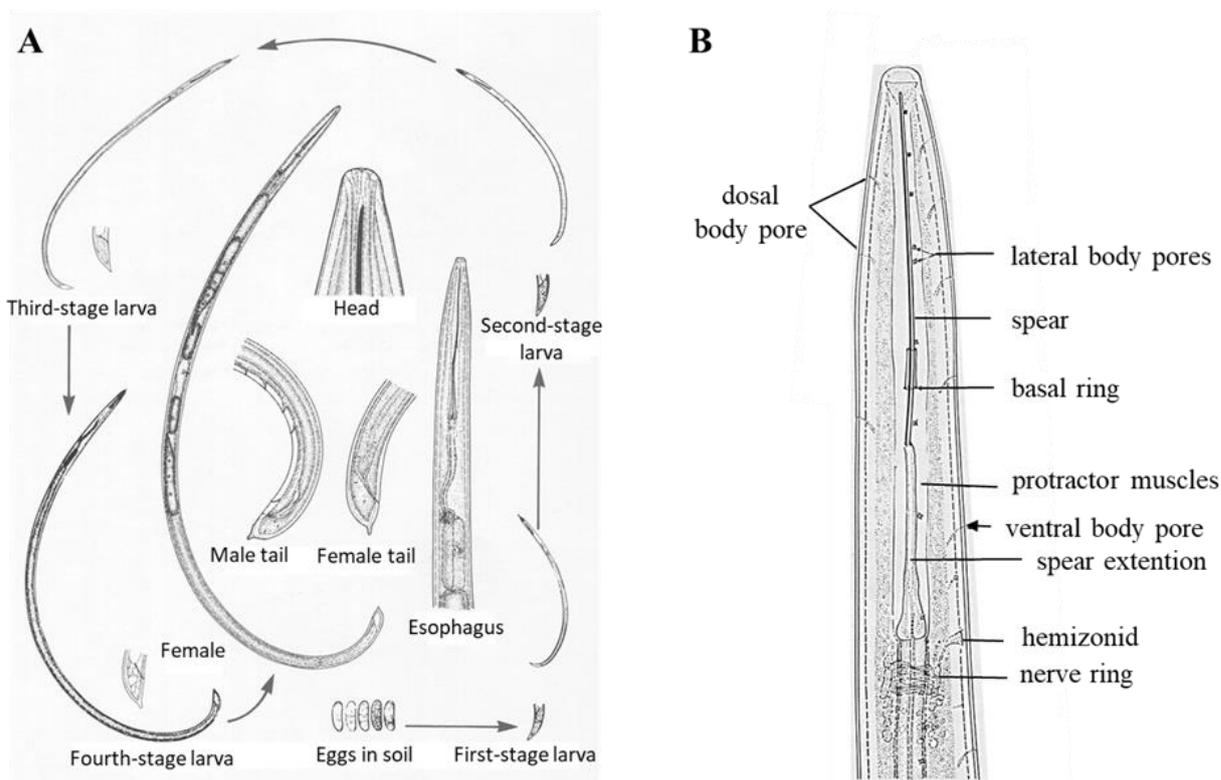


Figure 1.18. The dagger nematode *X. index*. A: life cycle. B: Anterior part of esophagus with spear (odontostyle) averaging 126 μm long and the spear extension (odontophore) with average 70 μm in length; A: Drawing by Nemaplex, University of California, Davis (<http://nemaplex.ucdavis.edu/Taxadata/G143s3.aspx>); B: Adapted from Siddiqi (1986).

Eggs are laid singly in the soil close to the feeding site (Weischer & Wyss, 1976), hatch a few days later to juvenile 1 (J1) and then molt 4 times to reach the adults stage (Fig. 1.18 A). Males are rare. Under controlled conditions, *X. index* requires approx. 3 months to complete its life cycle (Taylor & Raski, 1964; Wyss, 2014) but in the field the life cycle may last one to several years depending on biotic and abiotic factors (Weischer, 1975). Under adverse conditions, including low moisture, low temperature, and the absence of host plants, *X. index* undergoes a quiescent phase during which physiological functions are limited and development is interrupted (Antoniou, 1989).

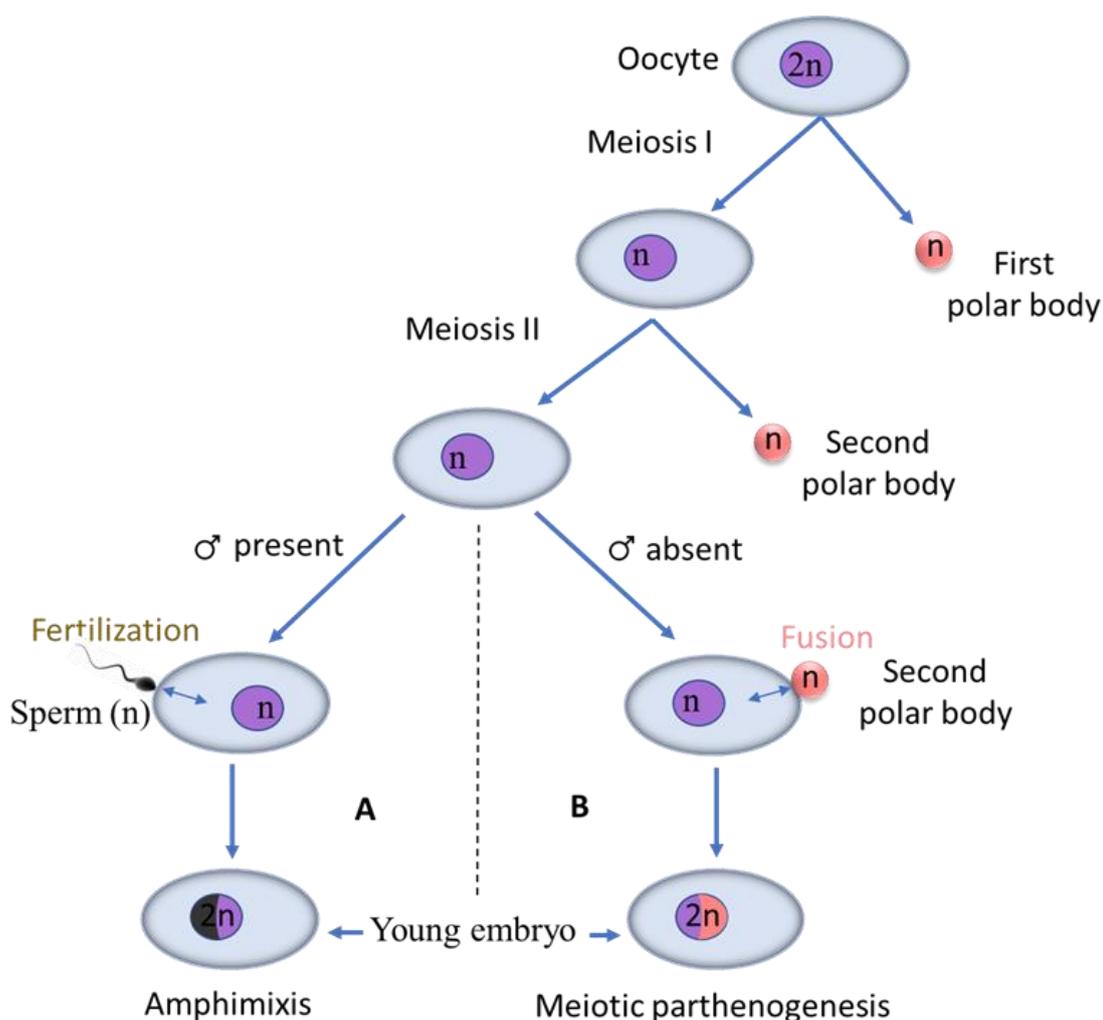


Figure 1.19. Schematic representation the reproduction modes of *X. index*. A: Amphimictic (sexual) reproduction mode (rare); B: Meiotic parthenogenetic reproduction mode (predominant). $2n$ represents the diploid chromosome number ($2n = 20$ in *X. index*) (adapted from Castagnone-Sereno et al., 2013)

Temperature is an important modulating factor on the reproduction and life cycle of *X. index*, which is typically associated with grapevines in warm climates. *Xiphinema index* has been shown to survive in a wide range of soil temperatures from -11°C to 35°C , but constant temperatures for 10 days of 45°C or -22°C killed the nematodes (Van Zyl et al., 2012). The *X. index* population increased more rapidly as the soil temperature increased from 16 to 28°C . According to Cohn & Mordechai (1969), the life cycle took 3 to 5 months to complete at 28°C , but slowed down to 7-9 months at $20 - 23^{\circ}\text{C}$. In Italy, it was found that *X. index* numbers are lower in winter (Coiro et al., 1987; Coiro & Serino, 1991). By contrast, in California the *X. index* populations peaked in the winter (Feil et al., 1997) possibly due to more accurate sampling in moist soils. In England, under controlled conditions, *X. index* egg-laying peaked during summer months and reached maximum populations in autumn (Siddiqi, 1986)

All stages, including adult females, move through the soil to find and feed on roots (Nicholas et al., 2007). *Xiphinema index* feeds near the root tips by inserting its stylet into the root tissues (De Klerk & Loubser, 1988). The time that *X. index* stays at one feeding site can vary from several minutes to several days. Root areas already fed on, attract more nematodes and can result in crowding (Weischer & Wyss, 1976).

Host plants and symptoms

Xiphinema index has a limited host range. *Vitis* spp. are known as the main host but fig (*Ficus carica*) is a better host plant than grapevines (Brown & Coiro, 1985). This justifies the use of fig as the preferred plant for nematode multiplication under controlled conditions. *Xiphinema index* has also been recorded as present on roses and citrus (Nicholas et al., 2007), on petunia, tomato, *C. amaranticolor* and tobacco (Coiro & Serino, 1991).

Xiphinema index not only causes indirect damage by GFLV transmission but also direct damage on grapevine root tips by inducing terminal swellings and necroses (Fig. 1.20). *Xiphinema index* feeding initially causes a swollen club-like gall on root tips. The feeding wound then becomes reddish brown to black and forms slightly sunken lesions on the roots (Wyss, 1978). Common symptoms of *X. index* feeding are plant stunting, chlorosis, galls forming and then root necrosis. This galling has been shown to occur as early as 24 hours after feeding (Fisher & Raski, 1967). The combined effect of *X. index* feeding and its association with GFLV may kill grapevines (Nicholas et al., 2007).

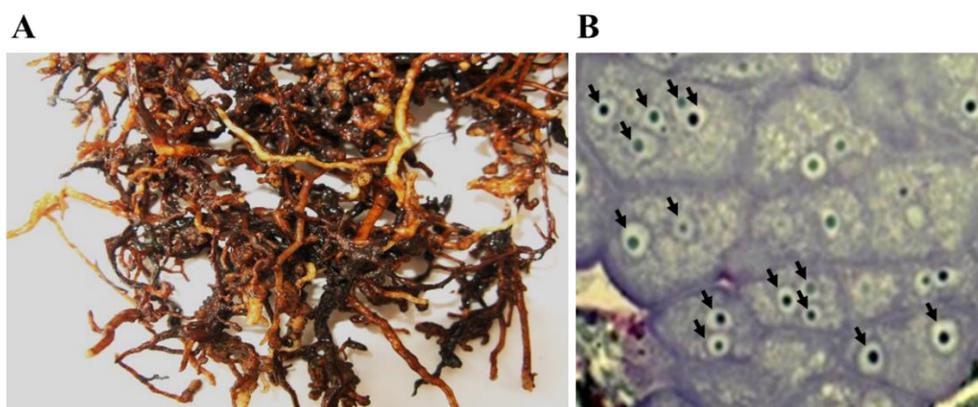


Figure 1.20. Direct damage on grape caused by *X. index*. A: Terminal swellings ('galls') on grapevine root system of the susceptible cultivar 'Rupestris du Lot', B: Longitudinal section through a swollen root tip of a young grapevine root showing multinucleate cells after *X. index* feeding. Nuclei are indicated by black arrows. (Photos credit: INRA Sophia Antipolis).

Distribution in the soil

In general, soil texture and structure are critical for the development of soil-inhabiting nematodes (Pitcher, 1975). Pore space and moisture capacity are physical characteristics that determine soil suitability as a habitat for nematodes. Nevertheless, *X. index* has been recovered from a wide range of soil types (sandy soil, silt and clay heavy soils, etc.). Therefore, its distribution is mostly limited by adequate moisture and the presence of suitable hosts. However, it prefers heavy soils that are less subject to drought but is also present in sandy soils if moisture is sufficient (Esmenjaud et al., 1992).

The horizontal and vertical distribution of *X. index* in vineyard soils is generally clustered and closely related to the distribution of roots. Its density is higher within than between vine rows (Feil et al., 1997), and the aggregated pattern of nematode distribution correlates with GFLV-infected grapevines (Villate et al., 2008). Usually absent from shallow soil layers, *X. index* is located 0.3–1.5 m deep in vineyard soils where fine rootlets are most abundant (Esmenjaud et al., 1992; Feil et al., 1997; Villate et al., 2008) (Fig. 1.21). Specimens have even been detected at a depth of 3.6 m (Raski et al., 1965).

The movement of *X. index* is limited to a few dozens of cm per year (Pitcher, 1975; Taylor et al., 1994). It moves horizontally and vertically in the soil by following the progression of the roots from which it feeds (Thomas, 1981). *Xiphinema index* are disseminated with the soil through the use of contaminated equipment such as tractors, planters, planting of infested plants/sods, and soil transfer. Additionally, *X. index* may be dispersed passively by water streams or irrigation canals (Rocuzzo & Ciancio, 1991).

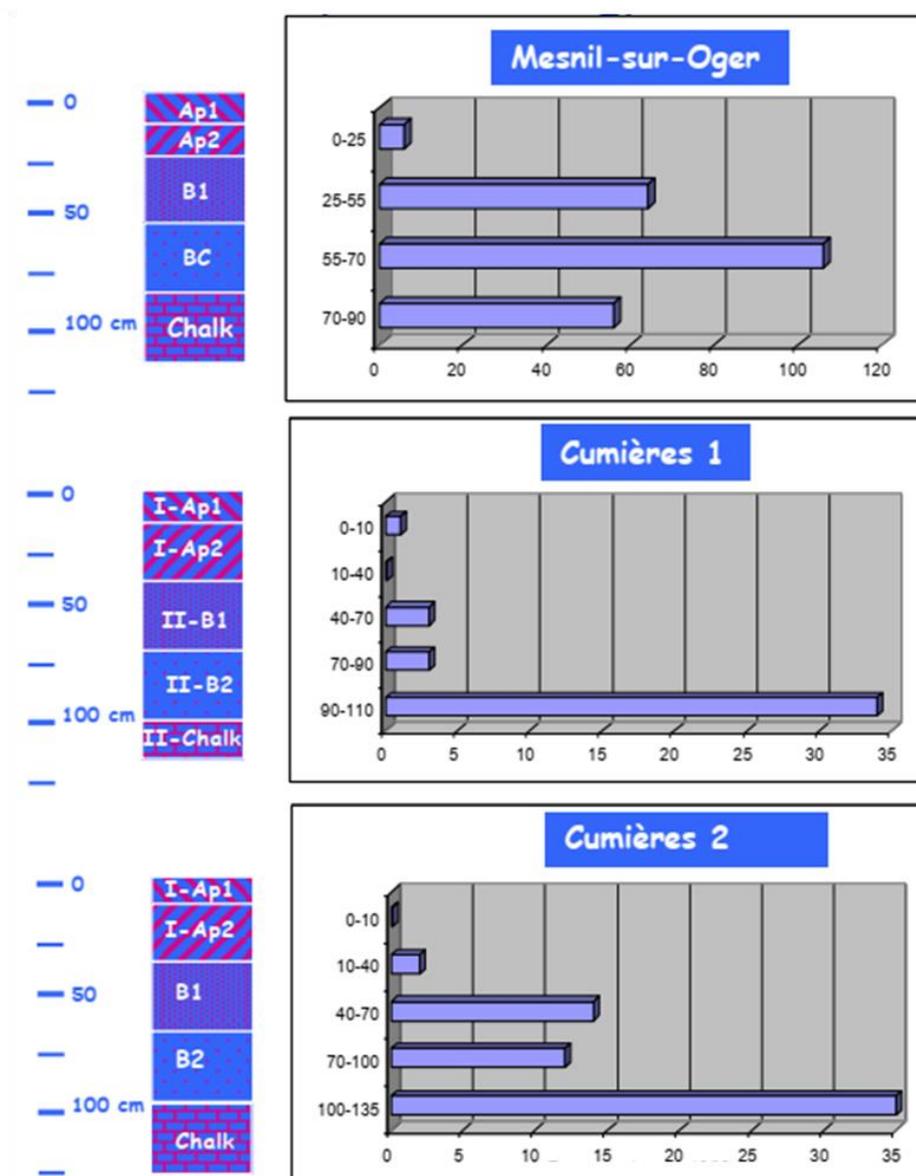


Figure 1.21. Densities of *X. index* in three soil profiles in Champagne vineyard (France) in different soil layers (depths of soil layers are indicated in cm on the Y axis and nematode numbers are indicated per kg of soil on the X axis) (Esmenjaud et al., 1992).

1.4.1.6. Genetic diversity at the field, local and regional scales

Genetic diversity of *X. index* has been studied at the field, local and regional scales using species-specific microsatellites (Villate et al., 2009). At the grapevine field scale, microsatellite markers showed no isolation by distance but a differentiation among sampling points within the grapevine field (Villate et al., 2010). This pattern is consistent with the clonal reproductive mode (rate of 95–100%) and the limited capacities of active dispersal of *Xiphinema* spp., leading to a patchy spatial distribution within grapevine fields (Villate et al., 2008; 2010). Moreover, a small number of hybridization events were identified at the overlapping zones between different genetic clusters, i.e. probably where two independent

nematode introduction events and their cognate patches came into contact (Villate et al., 2010). Villate et al. (2008) confirmed the preferred mode of nematode dissemination along the row in the grapevine field.

1.4.1.7. Interaction *X. index* – GFLV

Virus transmission

Hewitt et al. (1958) reported that the dagger nematode *X. index* is the vector of GFLV. Since then, this nematode has been considered as one of the most important viticultural pests in grape-growing areas throughout the world. In 1962, Hewitt et al. fulfilled Koch's postulates by using viruliferous *X. index* to transmit GFLV from *C. amaranticolor* to grapevines in which typical symptoms developed. These experiments demonstrated conclusively that GFLV is the causal agent of fanleaf degeneration disease.

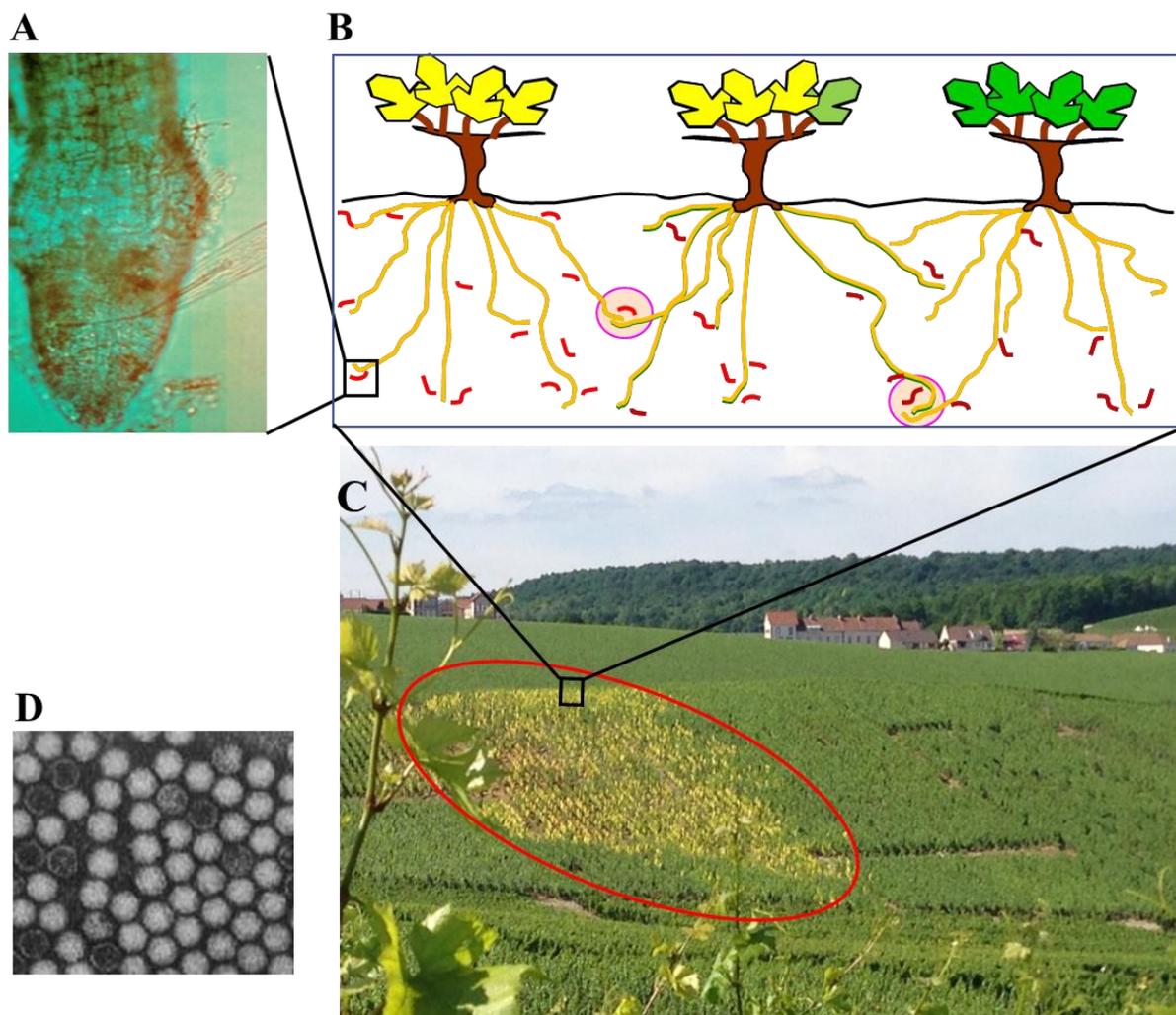


Figure 1.22. Dissemination scheme of *Grapevine fanleaf virus* by *X. index* in the field. **A:** Anterior part and stylet of a nematode feeding at the root tip. **B:** Scheme illustrating how the nematodes (in circles) vector the virus from plant to plant in the soil. **C:** GFLV field aerial symptoms (red ellipse) corresponding to a nematode focus in the soil. **D:** GFLV viral particles under electron microscope.

In 1963, typical symptoms of fanleaf degeneration were reproduced in grapevines following graft transmission from GFLV-infected *C. amaranticolor* and the virus was successfully transferred by sap transmission from symptomatic grapevines to *C. amaranticolor* (Dias, 1963). It is worth noting that this discovery was controversial in the early 1960's because phylloxera was believed to be involved in fanleaf degeneration.

Virus retention

The transmission of GFLV by *X. index* is characterized by a specific and complementary association (Brown & Weischer, 1998). *Xiphinema index* feeds on growing root tips and acquires GFLV particles upon feeding (Hewitt et al., 1958; Raski et al., 1983; Wyss, 2014). The transmission process is mediated by the ability of *X. index* to ingest GFLV particles from a virus source grapevine, retain virions at specific retention sites within its feeding apparatus, and subsequently infect a recipient vine by release of virus particles from the retention sites.

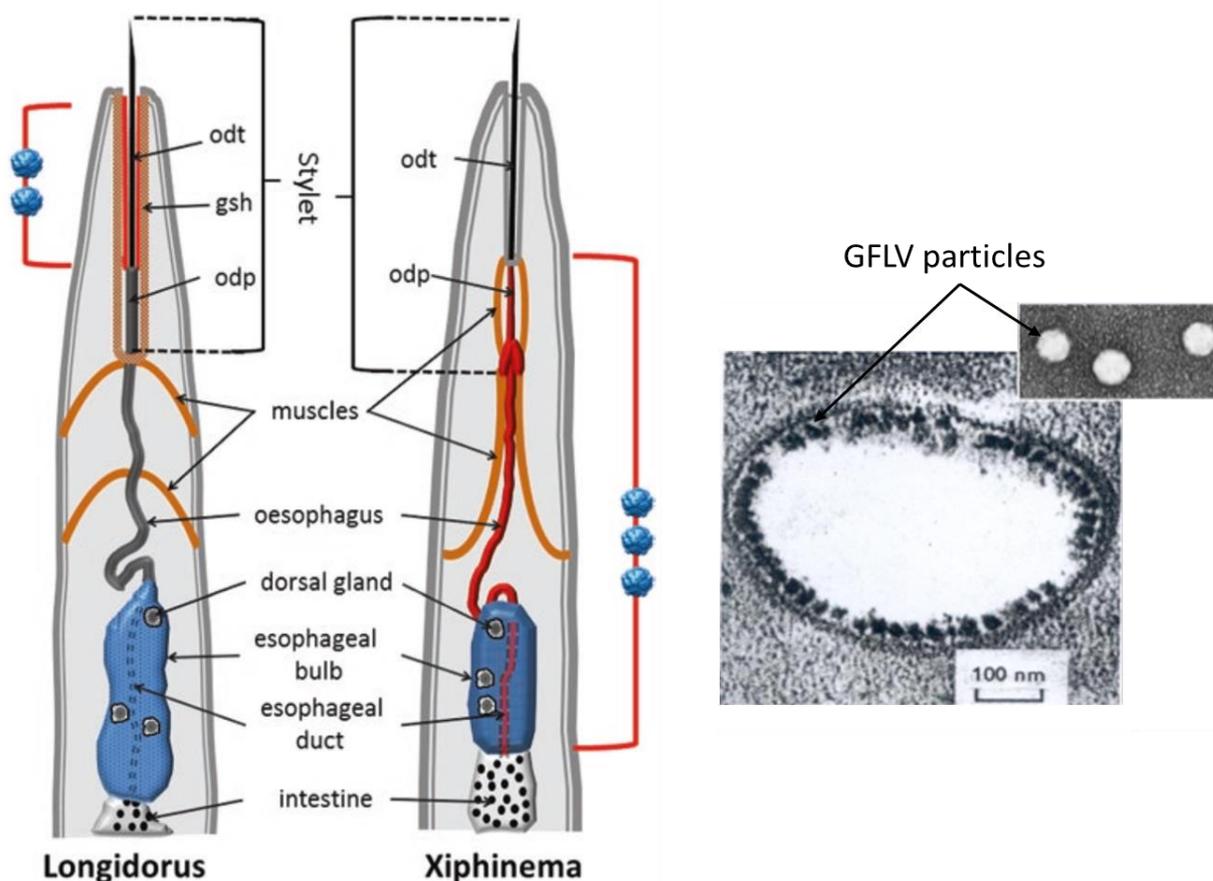


Figure 1.23. Schematic representation of the morphological structure of the anterior region of *Longidorus* and *Xiphinema* showing the feeding apparatus. The retention site of the viral particles is highlighted in red brackets for both genera; odp: odontophore, gsh: guiding sheath, odt: odontostyle (adapted from Andret-Link et al., 2017. GFLV particles photo: Taylor & Robertson, 1970)

In vector nematodes, viral particles are adsorbed at specific sites probably in association with a receptor present at the inner cuticle of the digestive system. The

Longidorus and the *Xiphinema* differ in their retention sites of viral particles. In *Longidorus* vectors, viral particles are present exclusively between the odontostyle and the "guide" membrane (Fig. 1.23) (Roberts & Brown 1980). In *Xiphinema* vectors, viral particles are distributed over a much larger segment of the food system (Fig. 1.23). This difference in location and retention area probably explains the difference in the storage time of viral particles between the two kinds of nematodes. After each moult, the viral particles are eliminated from nematodes. Therefore, nematodes are no longer viruliferous and must re-feed on an infected plant to recover the virus (Taylor & Robertson, 1970).

In 2005, Demangeat et al. showed that under starving conditions *X. index* can survive and retain GFLV for 48 months in controlled conditions at 7 and 20°C without host plants.

Specificity determinants

The *X. index*/GFLV interaction is very specific. GFLV is transmitted exclusively by this nematode which itself cannot transmit any other *Nepovirus* (Demangeat et al., 2010). The nematode-specific basis of *Nepovirus* transmission is uncertain. Different strains of *X. index* reproduce at different rates but did not differ in GFLV transmission competencies (Demangeat et al., 2010). The transmission specificity of GFLV maps to their respective 2C^{CP} (Andret-Link et al., 2004) and a 11 amino-acid region of the coat protein (in the β B- β C loop of the two-fold axis of the coat protein junction) determines transmission specificities of *X. index* (Schellenberger et al., 2010).

Dissemination of GFLV

The dissemination of GFLV on grapevine may be through different ways, such as its vector *X. index* (from infected plant to healthy plant during feeding (Fig. 1.22)), human activities (grafting and soil transfer) and seeds (occasional) (Andret-Link et al., 2004; Villate et al., 2010). The transfer of soil contaminated with viruliferous *X. index* and/or GFLV-infected plant material allows GFLV dissemination over short and long distances (Villate et al., 2008).

However, the main worldwide dissemination of GFLV is via grafting and extensive exchange of propagation material (Martelli, 1978). Despite rigorous sanitary controls, producers lack efficient technologies to control the infection since nematicide chemicals tend to be prohibited. Interestingly, GFLV is presumably native to *V. vinifera* (Hewitt, 1968; Raski et al, 1983) and probably originated from the same area as domesticated grapevines, i.e. the Caucasus regions between the Black and Caspian seas. GFLV is likely to have been disseminated from this center of origin throughout Europe and then to the rest of the World (Andret-Link et al., 2004).

Nevertheless, despite the implementation of effective measures to limit GFLV dissemination over long distances, the virus remains a recurrent problem in established diseased vineyards.

1.4.1.8. The integrated control of *X. index*

Control strategies against *X. index* aim to eliminate or reduce *X. index* numbers in the soil around the grapevine root system. The control of *X. index* must face two major spatial and temporal limiting factors. These are i) the location of the nematode in deep soil layers and ii) the survival of the nematode and of the virus in the nematode for over 4 years.

Chemical control

Until recently, *X. index* control strategies relied almost exclusively on soil disinfection to reduce *X. index* numbers and delay viral transmission. Before applying nematicides, it is recommended to pull out all the root system, carefully remove the root debris in deep soil layers and dry the soil as much as possible (Van Zyl et al., 2012). However, chemical treatments have a limited efficiency particularly in grapevine plots because they only diffuse in the upper soil layers (0 to 30-40 cm) and cannot reach the layers beyond where the highest nematode densities are usually found and feed on deep roots (Thomason & McKenry, 1975; Esmenjaud et al., 1992; Villate et al., 2008) (Fig. 1.21).

Chemical nematicides are being banned in an increasing number of countries, due to their adverse environmental effects (Abawi & Widmer, 2000)

Antagonistic fallow plants

An alternative is the use of plants with an antagonistic effect on *X. index* to be grown in the fallow period between two successive vine crops. Fallow crops such as marigold and hairy vetch have been shown to reduce *X. index* populations in the vineyard (Villate et al., 2012).

Rootstock resistance

To combat *X. index* in grapevine, resistant rootstocks are a promising perspective and this aspect will be discussed in 1.4.2 hereafter. This strategy has already long been the best way to control the root-knot nematodes in vineyards. In the US, resistance sources from *Vitis* spp. have been used early and the first RKN-resistant rootstocks were developed from *V. champinii*, a species which carries resistance to common field populations of *M. arenaria*, *M. incognita* and *M. javanica*. Nevertheless, this source was overcome locally by virulent populations of *M. arenaria* and *M. incognita*. This led to the selection of new hybrid material, mostly containing *R* genes from *V. champinii*, *V. mustangensis* or *M. rotundifolia* (Saucet et al., 2016)

1.4.2. The resistance against *X. index*

1.4.2.1. Resistance sources

There have been many researches on grapevine aimed to find out resistance sources to *X. index* and the subsequent resistance conferred to GFLV via the vector nematode, in greenhouse (Harris, 1983; Walker et al., 1991; Malan & Meyer, 1993; McKenry et al., 2004; Esmenjaud et al., 2010) and in field conditions (Walker, 1994). Host suitability towards *X. index* of some rootstock examples is reported in Table 1.6.

Resistance in *Vitis* spp.

No noticeable resistance has been found in the cultivated grapevine *V. vinifera*. The highest resistance to *X. index*, firstly searched in late 20th century (Harris, 1983), was found in accessions from *V. candicans*, *V. solonis*, *V. arizonica*, *V. rufotomentosa* and *V. smalliana*. Moderate resistance was observed in *V. riparia*, *V. rubra* and *V. slavinii* (Ollat et al., 2016). Among commercial rootstocks, 1613C, a complex hybrid involving *V. riparia* and *V. longii* in particular, showed moderate resistance and all others, including the *V. champinii* rootstock Salt Creek (Ramsey), were quite susceptible (Kunde et al., 1968). Harris (1983) also found 1613C moderately resistant. Malan & Meyer (1993) with a South African population of *X. index* confirmed the resistance of 1613C and the susceptibility of Ramsey. However, ELISA tests detected the presence of GFLV in the roots of all cultivars after four months and the systemic spread of the virus from six to 18 months after inoculation. There was no evidence of resistance to the transmission of GFLV through feeding of *X. index* in any of the rootstocks studied, even though some had a low reproduction potential for the nematode and no root damage was observed. Boubals & Pistre (1978) concluded similarly when, five years after inoculation, all the accessions tested showed foliar symptoms of GFLV. They confirmed the susceptibility of Salt Creek, but not the resistance of 1613C, and showed a moderate level of resistance in *V. riparia* 'Gloire de Montpellier'. The results of Boubals and Pistre showed that great differences of susceptibility/tolerance could exist among varieties of the same *Vitis* species. For example, *V. riparia* 'Gloire de Montpellier' is moderately resistant but *V. riparia* 'Messner n°9' is very susceptible. The evidence of poor correlation between the resistance to transmission of GFLV and the resistance to nematode feeding in *Vitis* species was also observed by Staudt & Kassemeyer (1990). Though the susceptibility of *V. cinerea* to *X. index* was reported by Kunde et al. (1968) and Boubals & Pistre (1978), a high resistance of the rootstock cultivar 'Börner' (*V. riparia* x *V. cinerea*) to nematode feeding and GFLV infection was claimed (Becker & Sopp, 1990) but is controversial (Sopp et al., 1998; Ipach et al., 2000).

Table 1.6. Host suitability to *X. index* of some grapevine rootstocks. R, resistant; MR, moderately resistant; S, susceptible

Rootstock	Genetic origin	Host suitability	Reference
Harmony	(<i>V. longii</i> x Othello) x Dog Ridge	R	Harris (1983)
Freedom	(<i>V. longii</i> x Othello) x Dog Ridge	R	
3309	<i>V. rupestris</i> x <i>V. riparia</i>	R	
1613C	<i>V. longii</i> x [<i>V. vinifera</i> x (<i>V. riparia</i> x <i>V. labrusca</i>)]	R	
VR 039-16	<i>V. vinifera</i> x <i>M. rotundifolia</i>	R	Walker et al. (1991) McKenry et al. 2004)
VRH 8771	<i>V. vinifera</i> x (<i>M. rotundifolia</i> cv. NC184-4)	R	Esmenjaud et al. (2010)
Nemadex Alain Bouquet (RPG1)	VRH 8771 x (<i>V. Berlandieri</i> x <i>V. rupestris</i> cv. 140Ru)	R	
NC 35-50	T6-38op x <i>M. rotundifolia</i>	R	
Riparia Gloire de Montpellier (RGM)	<i>V. riparia</i>	MR	
Schwarzmann	<i>V. riparia</i>	MR	Harris (1983)
110R	<i>V. berlandieri</i> x <i>V. rupestris</i>	S	Malan & Meyer(1993)
Ramsey	<i>V. champinii</i>	S	Ambrosi (1967)
Dog Ridge	<i>V. rupestris</i> x <i>V. candicans</i>	S	
Jacquez	<i>V. aestivalis</i> x <i>V. cinerea</i> x <i>V. vinifera</i>	S	
775 Paulsen	<i>V. berlandieri</i> x <i>V. rupestris</i>	S	
VRH 8624 (T6-38)	<i>V. vinifera</i> x <i>M. rotundifolia</i>	S	Esmenjaud et al. (2010)
Rupestris du Lot (RL)	<i>V. rupestris</i>	S	

In France, the rates of GFLV infection of scions of Cabernet-Sauvignon grafted on Börner and the susceptible rootstock SO4 were equivalent six years after planting (A. Bouquet, unpublished) (see experiment in Domaine du chapitre in Chapter 4, part 2). More

recent screening for resistance in clonal accessions of the species *V. aestivalis*, *V. amurensis*, *V. bayleyana*, *V. berlandieri*, *V. bicolor*, *V. candicans*, *V. cinerea*, *V. doaniana*, *V. flexuosa*, *V. lincecumii*, *V. piazeski*, *V. rubra*, *V. rupestris*, and *V. simpsonii*, did not allow to detect *X. index*-resistant plant material (Esmenjaud et al., 2010). By contrast, the accessions 'Grand Glabre' and '10128' in *V. riparia* exhibited a good level of resistance in recent evaluations (D. Esmenjaud & N. Ollat, unpublished). Interestingly in an accession of the species *V. arizonica*, a resistance was characterized in UC Davis and shown to be monogenic (Xu et al., 2008). By contrast, another accession, *V. arizonica* cv. Wetmoore, resulted quite susceptible (D. Esmenjaud & N. Ollat, unpublished).

Resistance in *Muscadinia rotundifolia*

Muscadines (*M. rotundifolia*) have an overall high degree of resistance to pests and diseases, especially Pierce's disease which severely limits production of *Vitis* species in the south-eastern United States (Bouquet, 1980). They are also among the most resistant grape species to the phylloxera aphid, *D. vitifolia*, which causes devastating root injury to *V. vinifera* (Husmann & Dearing, 1916).

Muscadine grapes comprise currently the best potential sources for *X. index* resistance and induce a delay in GFLV transmission (Bouquet, 1981; Bouquet & Danglot, 1983). The genus *Muscadinia* has a chromosomal number ($2n = 40$) different from the genus *Vitis* ($2n = 38$). Consequently, introducing this resistance into *Vitis* material by hybridization has been difficult. Despite this limitation, a few resistant intergeneric F1 hybrids *V. vinifera* x *M. rotundifolia* have been obtained in France (Bouquet, 1980) and the US (Walker et al., 1991). In the US (UC Davis breeding program), these were the *V. vinifera* x *M. rotundifolia* (VR) hybrids, VR039-16 and VR043-43 (Table 1.6), both patented and released as rootstocks highly resistant to *X. index* and preventing fanleaf degeneration. However, in the field these resistant rootstocks showed resistance to *X. index* feeding but they had no ability to inhibit the transmission of GFLV by its vector nematode. Nevertheless, although scions grafted onto these rootstocks became infected with GFLV, disease was not expressed (Walker et al., 1994a; 1994b).

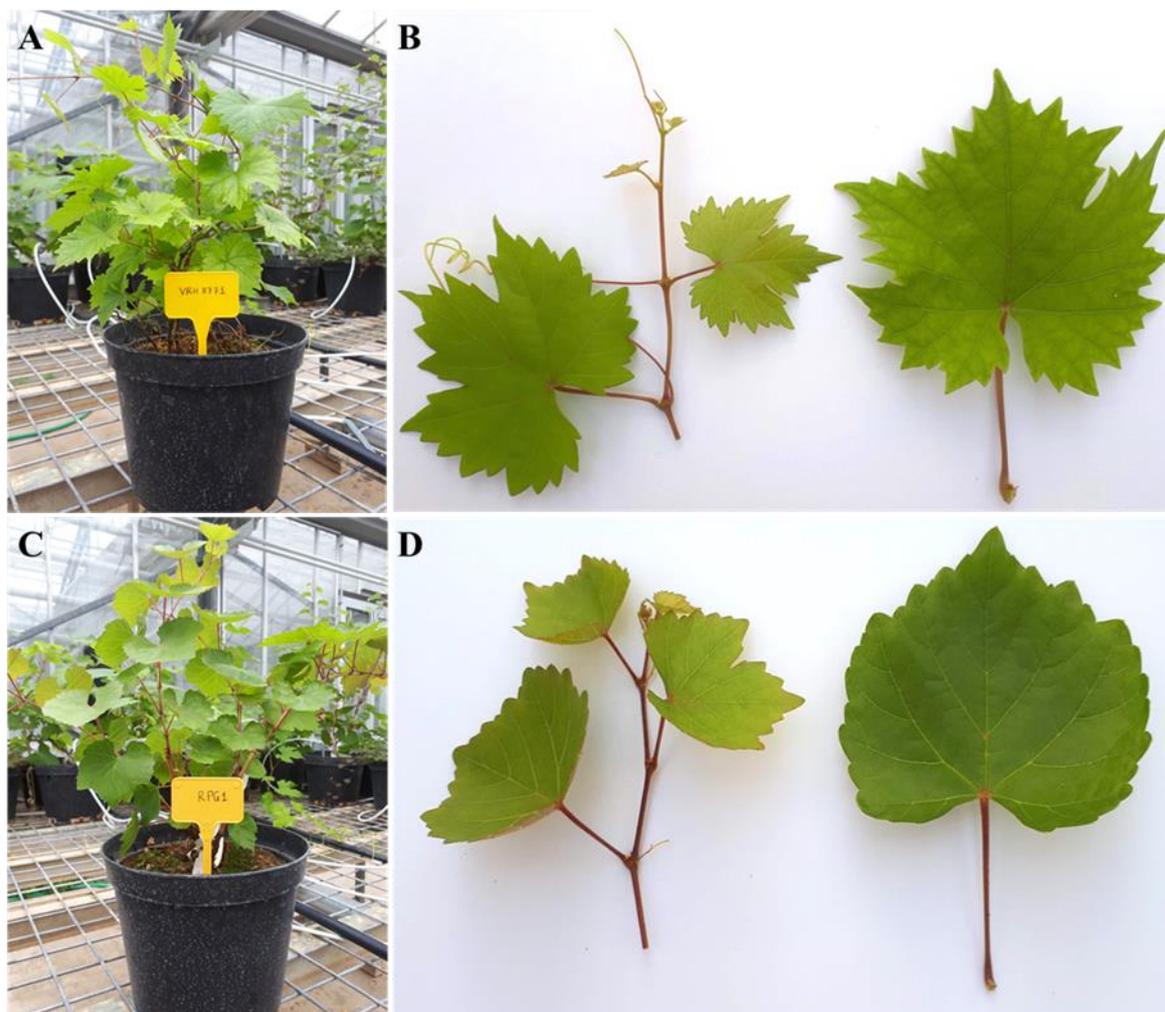


Figure 1.24. The two resistant clones used in the greenhouse experiment of Chapter 3. Upper photos: VRH8771. A: whole plant, B: shoot and leaf. Lower photos: RPG1. C: whole plant, D: shoot and leaf.

In France, the F1 hybrid *V. vinifera* x *M. rotundifolia* VRH8771 (VRH for *Vitis* x *rotundifolia* hybrid) (Fig. 1.24 A&B) is a good source for resistance to *X. index* (Esmenjaud et al., 2010). An individual called RPG1 and obtained by backcrossing VRH8773 (a brother clone of VRH8771 which is no longer available) with the *Vitis* rootstock accession 140Ru has shown good resistance and is now registered as a new rootstock (called ‘Nemadex Alain Bouquet’) (Fig. 1.24 C&D; Fig.1.26) to be used in highly nematode-infected plots under certain soil conditions (Bouquet et al., 2000; 2003; Esmenjaud et al., 2014) (Table 1.6). Considering that American VR039-16 and VR043-43 rootstocks have been practically removed from the market, Nemadex Alain Bouquet is currently the sole commercial rootstock which is resistant to *X. index* and delays GFLV (Ollat et al., 2011).

1.4.2.2. Histological mechanisms of resistance

Preliminary histological studies, conducted on resistant muscadine material (Staudt & Weischer, 1992), showed that this species displays a hypersensitivity phenomenon. At INRA-ISA, these observations were refined on the roots of the resistant *Muscadinia* F1 hybrid

VRH8771 and of the resistant BC1 cross RPG1: attacks induce a hypersensitive-like response with no or limited galling while the susceptible plants form galls in which multinucleated cells are observed (D. Esmenjaud and M. Banora, unpublished) (Fig. 1.25). Therefore, this resistance phenomenon presumably acts as a filter that prevents or at least delays transmission of virus particles to the healthy tissues.

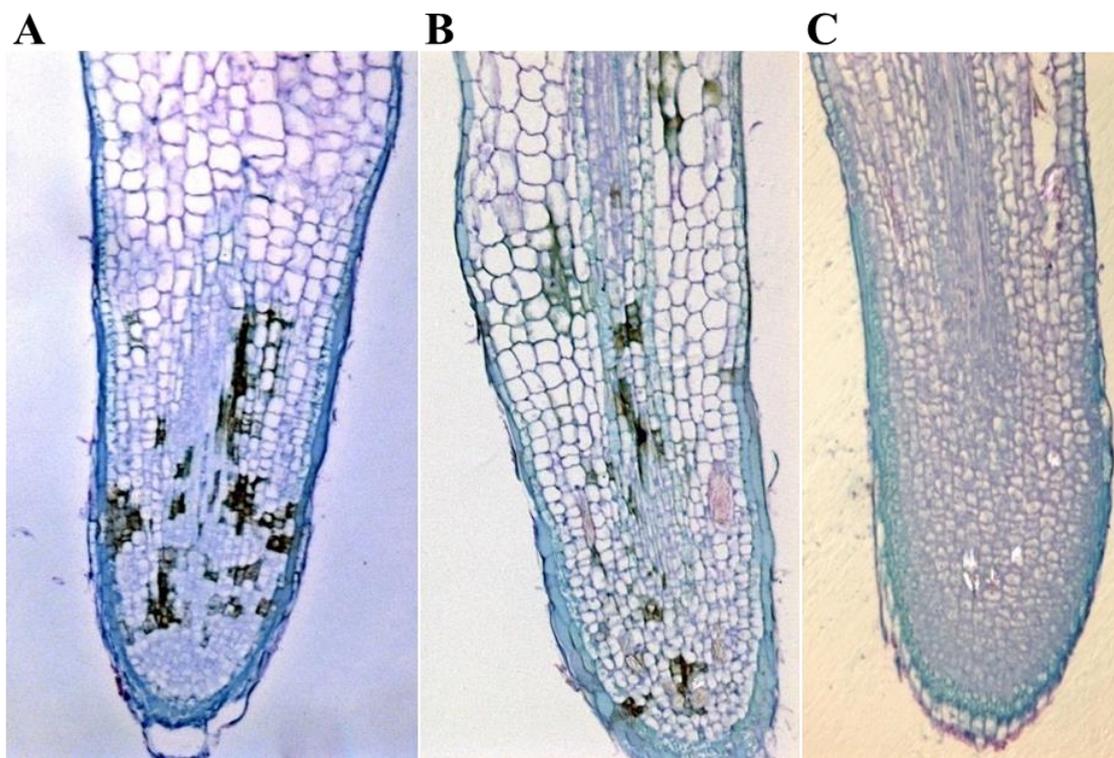


Figure 1.25. Histological responses of some *Muscadinia*-derived material resistant to *X. index* at 12 days post-inoculation. (A) VRH8771 and (B) RPG1 roots display hypersensitive-like responses. (C) Uninoculated control, RPG1.

1.4.2.3. Bases of the INRA breeding project for new *Muscadinia*-derived resistant rootstocks

Despite being a good source for *X. index* resistance, *M. rotundifolia* is not suitable as rootstock because of its poor rooting ability and its graft incompatibility with *V. vinifera* (Bouquet, 1980). Most but not all *V. vinifera* x *M. rotundifolia* hybrids (VRH) are resistant to *X. index* (Walker et al., 1994; Esmenjaud et al., 2010). As illustrated in Table 1.7, F1 hybrid material ranges from resistant (VRH 8771, VR 039-16) to susceptible (VRH 8624). When present in F1, the resistance can be inherited in the back cross 1 (BC1) level as in RPG1 (Esmenjaud et al., 2010).

As reported previously, in France, breeding for *Muscadinia*-derived resistant rootstocks was undertaken by Alain Bouquet approx. thirty years ago (Bouquet, 1980; Bouquet & Danglot 1983) and the first achievement has been the selection and the release of

the BC1 rootstock Nemadex Alain Bouquet (Nemadex AB or RPG1) (Ollat et al., 2011). The project is now being continued at INRA-EGFV in Bordeaux and, considering the limited viticultural characteristics of this rootstock (Claverie, 2017), its objective is to breed new *Muscadinia*-derived rootstocks that will carry i) resistances to both Phylloxera and *X. index* and ii) an adaptation to the wide range of pedoclimatic conditions of the French vineyards. This material will be selected in particular for good rooting ability and vigor, both features expected from its *Vitis* parentage. Proof-of-concept studies based on F1 and BC1 *Muscadinia*-derived materials already obtained have illustrated the ability of these resistant accessions to reduce considerably the vector nematode multiplication and to delay the cognate transmission of GFLV (Esmenjaud et al., 2010; 2011; 2014). The *Muscadinia-Vitis* F1 hybrid VRH 8771 is the resistance source retained in the current INRA breeding project. The detailed pedigrees of the VRH 8771 and RPG1 resistant materials, derived from the resistant *M. rotundifolia* accession NC 184-4, and their detailed male/female status are shown in Fig. 1.26.

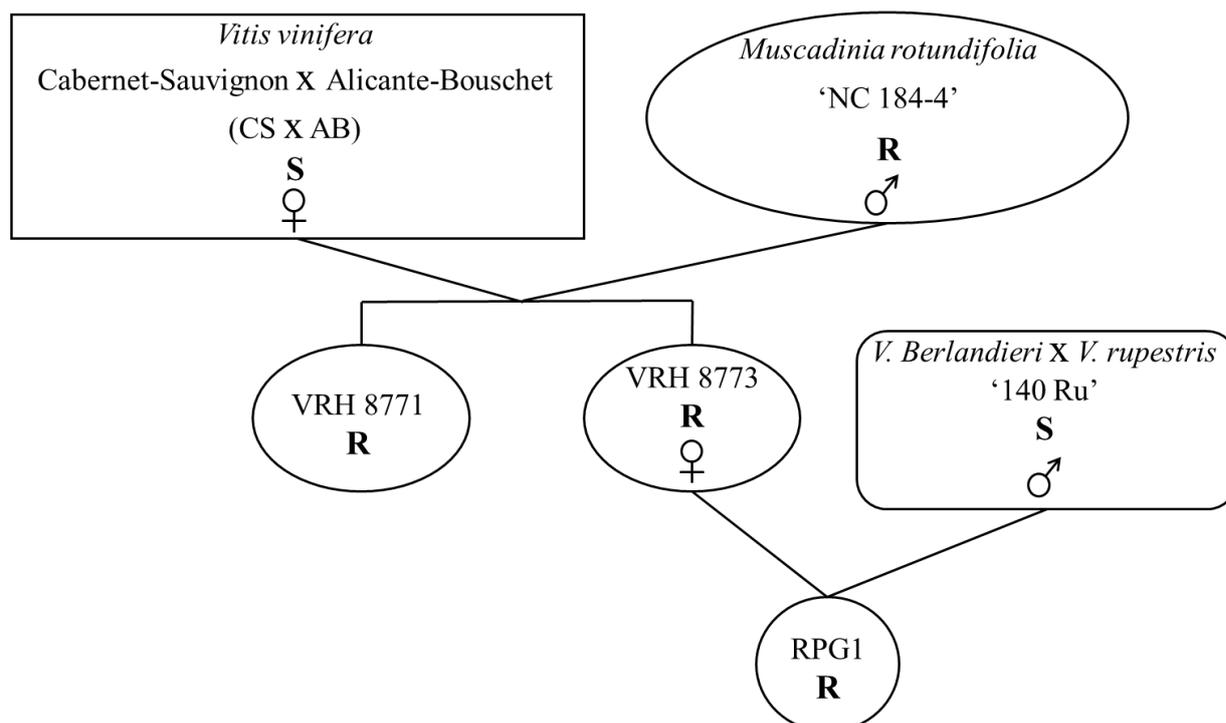


Figure 1.26. Pedigree of the resistant plant accessions derived from the muscadine resistance source NC 184-4. R, resistant; S, susceptible; RPG1 = Nemadex Alain Bouquet.

1.4.2.4. State of the art on the studies for resistance to *X. index* conducted at INRA UMR ISA

Resistance sources

Resistance studies conducted at INRA UMR ISA have considered several sources currently available at INRA-EGFV in Bordeaux (Table 1.7).

Table 1.7. *Vitis-Muscadinia* accessions available at INRA-EGFV, their host suitability to *X. index*, and their parentage. *Vitis-Muscadinia* and *Vitis* susceptible control materials are also shown.

Cross or species	Accession (% <i>Muscadinia</i>)	Host suitability	Female parent	Male parent
Intergeneric material				
<i>V. vinifera</i> x <i>M. rotundifolia</i>	VRH 8624 (T6-38) (50 %)	S	F2-35 (Carignan x CS)	<i>M. rotundifolia</i> Trayshed
	VRH 8771 (50 %)	R	42-36 (CS x AB)	<i>M. rotundifolia</i> NC 184-4
	VRH 97-99-79 (50 %)	R	42-28 (CS x AB)	<i>M. rotundifolia</i> Carlos
	NC 35-50 (37.5 %)	R	T6-38 OP seedling	<i>M. rotundifolia</i> Dixie
(<i>V. vinifera</i> x <i>M. rotundifolia</i>) x <i>Vitis</i> rootstock	RPG1 (25 %)	R	VRH 8773 [=(CS x AB) x NC 184-4]	<i>V. Berlandieri</i> Rességuier II x <i>V. rupestris</i> du Lot = 140Ru
<i>Vitis</i> material				
<i>V. riparia</i>	Gloire de Montpellier	MR		
<i>V. rupestris</i>	du Lot (RL)	S		
<i>V. vinifera</i>	Cabernet- Sauvignon	S		

R, resistant; S, susceptible; MR, moderately resistant; CS, Cabernet-Sauvignon; AB, Alicante-Bouschet

These resistance sources are F1 or more complex *Muscadinia-Vitis* intergeneric accessions because their muscadine parents are no longer available. Among these sources, the breeding program has been based and will continue to be based in priority on VRH 8771 as an F1 representative of the original parental muscadine accession NC 184-4. Several studies, presented hereafter, are being conducted to characterize the resistance properties of VRH 8771 together with RPG1 as a BC1 representative. Diverse control or reference materials have also been used: the susceptible *Muscadinia-Vitis* F1 VRH 8426, from the parental muscadine Trayshed, the susceptible *Vitis* accessions, *V. rupestris* du Lot and *V. vinifera* Cabernet-Sauvignon, and the moderately resistant accession *V. riparia* Gloire de Montpellier.

Durability of the muscadine-derived resistance to *X. index*

A preliminary study under greenhouse-controlled conditions has been conducted until 2010 (Esmenjaud et al., 2010). It considered the *Muscadinia-Vitis* F1 accession VRH 97-99-79 from the resistant muscadine accession Carlos (Table 1.7) and the BC1 representative RPG1, with *V. rupestris* du Lot and *V. riparia* Gloire de Montpellier as *Vitis* controls. Plant material has been obtained from *in vitro*, acclimatized in growth chamber for 2 months without hormones and grown in the greenhouse for 5 months before inoculation. An isolate created from a single female of the population *X. index* Fréjus has been used as inoculum. The reproduction factor has been evaluated after 1 and 4 years. As expected from previous evaluations, the F1 and BC1 materials resulted resistant ($RF \leq 1$) after one year. Nevertheless, the RF values for both accessions were significantly higher after 4 years (5.27 in VRH 97-99-79 and 7.57 in RPG1) than after 1 year. These values suggested that the nematode might have adapted progressively to the resistances carried by both accessions (Fig 1.27). If we hypothesize that their respective muscadine sources, Carlos and NC 184-4, confer an equivalent level of resistance, the higher RF value (even though it is not significant) in the BC1 than in the F1 appears logical.

The results from Esmenjaud et al. (2010) suggest, at least in the experimental conditions based on plants obtained from *in vitro*, an erosion of resistance over years. The limited durability of the resistance evidenced at both F1 and BC1 levels merits further investigations.

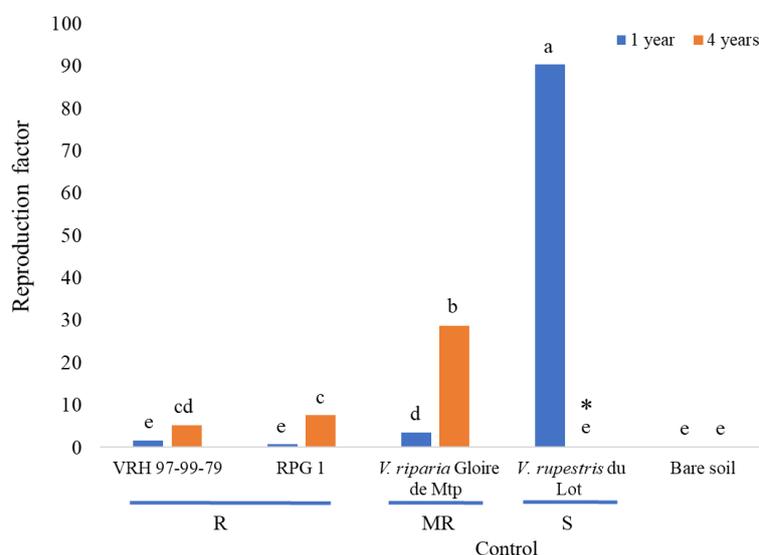


Figure 1.27. Reproduction factors (RF) of *X. index* in VRH 97-99-79 (= *Vitis* x *Muscadinia* F1 accession), in RPG1 (= F1 backcrossed by the *Vitis* rootstock 140Ru), and in two reference control rootstocks *V. rupestris* du Lot (RL) and *V. riparia* Gloire de Montpellier (RGM), evaluated after 1 and 4 years. For these data (6 replicates), bars with at least one common letter do not differ according to Fisher's (LSD) multiple range test at $p \leq 0.05$. Plant material has been obtained from *in vitro* plants. * At 4 years in *V. rupestris*, values are lacking because of plant mortality consecutive to very high attacks after 1 year. R resistant, MR moderately resistant, S susceptible (adapted from Esmenjaud et al., 2010).

Inheritance of resistance to *X. index* in muscadine

Previous results highlight the need to decipher the genetics of resistance to *X. index* in muscadine. To alleviate this concern, a greenhouse study on the inheritance of resistance carried out by the muscadine source NC 184-4 has been undertaken. Despite the difficult hybridization between the genera *Vitis* and *Muscadinia*, crosses repeated over several years have allowed to obtain a backcross (BC1) progeny of 66 individuals between VRH 8771 and the susceptible (S) *V. vinifera* cv. Cabernet-Sauvignon (CS). Resistance (R) phenotyping using a one-year test for the selection of major R factors showed a 58 R : 8 S segregation. The $\sim 7 R : 1 S$ ratio suggests the hypothesis of three dominant and independent R factors inherited from the muscadine source NC 184-4. Using a VRH8771 x CS genetic map in progress based on microsatellite and GBS (genotyping by sequencing) markers, a mapping method derived from BSA (bulked segregant analysis) has identified markers linked to resistance at three chromosomal locations that fit this hypothesis (Lalanne-Tisné et al., 2016). Nevertheless, given the low numbers of available BC1 individuals, complementary studies are needed. Next studies will aim at confirming the polygenic hypothesis by assessing the segregation of each putative single factor in new BC2 progenies. This will be tentatively performed by backcrossing appropriate BC1 individuals putatively carrying a single factor with S (recessive) individuals. Another study to be conducted consists in evaluating the effect of the R factors on GFLV infection. Segregating individuals representative of the diverse putative combinations of factors will be evaluated for the dynamics of their viral infection under a high pressure of viruliferous *X. index* nematodes. In this thesis work, it is important to retain the information that resistance appears polygenic and would be conferred by major dominant gene

I.5. STUDY PRESENTATION

I.5.1. Context and originality

The overall objective of my study is to get a better knowledge of the genetic variability of *X. index* in order to consider it in new control approaches. I will first try to increase our knowledge of the nematode genetic diversity at the world scale by a phylogeography approach. Taking into account these data, my second objective will be to assess the durability of the resistance to *X. index* and the cognate putative adaptation of the nematode facing this plant resistance.

I.5.2. Study objectives

I.5.2.1. Worldwide genetic diversity of the grapevine vector nematode

As the geographical distribution of the variability is key information for diversity studies, my work will aim at determining the native area of *X. index* and at tracing its dissemination routes. Four mitochondrial markers (*CytB*, *ATP6*, *ND4* and *CO1*) completed by 8 microsatellite markers will be used as tools to characterize the genetic diversity at the world scale.

My results should contribute to answer the question: ***How is shaped the worldwide genetic diversity of X. index?***

I.5.2.2. Application to the development of the resistance strategy

As already mentioned, females reproduce by meiotic parthenogenesis but occasional sexual reproduction may also occur even though males are rare (Villate et al., 2010). The reproductive status of the nematode widely affects the nematode variability. In the aim of an optimal use of the resistance strategy based on *Muscadinia*-derived resistant rootstocks, my objective will be to establish whether the long-life cycle of grapevine resistant material will allow the nematode to adapt over years and thus compromise its control. This question is particularly accurate given that preliminary data suggest a progressive adaptation of plant material obtained from *in vitro*. I will thus study the plant resistance and the cognate nematode adaptation in classical plant material obtained from cuttings together with plant material obtained from *in vitro*. For this, several pure and traceable nematode lines created in our lab (chosen as representative of the diversity highlighted in a preliminary phylogeography study) have been mixed to challenge plant resistance. In order to monitor the putative nematode

adaptation, diverse accessions representing different levels of resistance will be evaluated.

This monitoring will aim at characterizing i) whether the nematode adapts and what is the link between this adaptation and the plant resistance status and ii), in the positive case, how this adaptation might take place: dominance of a particular line, occurrence of hybridization phenomena...

My results should contribute to answer the question: ***Does the nematode X. index adapt and, if yes, how does it adapt to resistant grapevine rootstock plants over years?***

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CHAPTER 2

CHARACTERIZATION OF THE WORLDWIDE *XIPHINEMA INDEX* GENETIC DIVERSITY

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CHAPTER 2: CHARACTERIZATION OF THE WORLDWIDE *XIPHINEMA INDEX* GENETIC DIVERSITY

II.1. INTRODUCTION

Xiphinema index is present in most vineyards over the world. In the wine industry and in other grape productions like fruit, raisin or juice, *X. index* is one of the top ten nematode pests together with root-knot nematodes and cyst nematodes. It not only causes direct damage on root tips by forming galls during its feeding but also transmits GFLV which is the major virus causing grapevine degeneration or 'Court-noué'. In France, this disease was recorded in around 70% of the investigated vineyards. A better knowledge of *X. index* variability at the individual and population levels will be very helpful for control strategies and for the evaluation of the resistant plant material to this nematode in particular. Hypotheses on the expansion of *X. index* from its putative 'native area' in the Eastern Mediterranean to the today's grape growing regions along with its host plant have been proposed but they lack reliable supporting data. Domestication of grapevine is dated from 5400-5000 BCE supported by archeological discoveries of ancient wine (from the tartaric acid detected on the inner surface of amphoras) together with fossils of grape seeds (from Caucasus, Armenia and Iran mountains). Then domesticated grapevine was disseminated East-to-West by different ancient civilizations such as the Hittites, Phoenicians, Greeks, Romans, and Arabs, etc., through the Middle East, the Near East, the central and Western Mediterranean until Italy, Southern France, and the Iberian Peninsula. Along with the dispersion of its main host, deciphering the Antiquarian spread of *X. index* up-to Western European vineyards is a challenging task.

The expansion of the domesticated vine to the rest of the world is more recent. The vine was established and spread to the 'New World' (North and South America) in the 16-17th centuries by the Spanishs. In North America, the local wild *Vitis* species (*Vitis* spp. and *Muscadinia*) had no wine potential and European vine plants were therefore introduced on the East coast of the United States at the end of the 17th century. One remarkable event was the accidental introduction, in the years 1858- 1860, of the phylloxera from the United States into Europe. This root aphid decimated the French and then the European vineyard in a few decades. In the fight against this pest, many non-*vinifera* *Vitis* species (out of which several ones were completely resistant) were also introduced from the US to Europe, to be used directly or after hybridization as resistant rootstocks.

In this chapter, we are going to use different sets of markers to study the genetic diversity of *X. index* at the world scale. The samples used originate either from the INRA-ISA collection or have been obtained during this study through our nematologists network.

The objective of this work is to:

- get a better knowledge of the genetic diversity of *X. index* at a world scale by using complementary markers: mitochondrial (maternal) genes and microsatellite (nuclear) sequences. We will also consider a few populations of *X. diversicaudatum*, a species very close to *X. index* but with a sexual reproduction mode, in order to compare the diversity of these two major *Xiphinema* species.
- use, as much as possible, an array of samples representative of the *X. index* geographic distribution in order to trace the dissemination routes of the nematode from its native area and recover the major historical events that paved its dispersal from the Antiquity epoch.
- establish which are the populations to retain as representatives of the global nematode diversity for the evaluation of the control strategies of *X. index* in the vineyards. In particular, this will allow conducting reliably the evaluation of the resistant material.

The manuscript written from this work has been submitted to the Journal 'Scientific Reports'.

II.2. SUBMITTED MANUSCRIPT

1 **Phylogeography of the soil-borne vector nematode *Xiphinema index*** 2 **sheds light into its Eastern origin and its dissemination with** 3 **domesticated grapevine**

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14 **ABSTRACT**

15
 16 The phylogeographical study of the soil-borne grapevine virus vector nematode
 17 *Xiphinema index* used mitochondrial and microsatellite markers of 82 samples from
 18 its worldwide distribution area. As expected in this mainly parthenogenetic species,
 19 the mitochondrial marker *CytB* exhibited a lower intraspecific divergence than the
 20 related amphimictic species *X. diversicaudatum*. In *X. index*, concatenated sequences
 21 of mitochondrial genes *CytB*, *ATP6*, *CO1* and *ND4*, displayed two clades divided each
 22 in two subclades. Three subclades grouped Eastern Mediterranean, Near- and Middle-
 23 Eastern samples while the samples from Western Mediterranean, Europe and the
 24 Americas all belonged to the fourth subclade. The highest polymorphism found in one
 25 of the Middle- and Near-East subclades strongly suggested that it contained the
 26 nematode native area. East-to-west nematode dissemination appeared to match the
 27 route of its domesticated grapevine host during Antiquity mainly by the Greeks and
 28 Romans. The Western subclade only comprised two close mitochondrial haplotypes.
 29 The first haplotype, from Southern Iberian Peninsula, Bordeaux and Provence
 30 vineyards, exhibited a high microsatellite polymorphism presumably due to ancient
 31 times introductions. The second haplotype contained a highly predominant
 32 microsatellite genotype widespread in Western countries and we hypothesized that its
 33 wide distribution is consecutive to recent dispersal during massive grapevine replants
 34 following the 19th-century phylloxera crisis. Taken together, our data allowed drawing
 35 a global picture of *X. index* diversity.

37 **INTRODUCTION**

38
 39 More than 4,100 species of plant-parasitic nematodes have been described¹ causing an
 40 economical damage of approx. \$US 80 billions per year². Among them, the dagger nematode
 41 *Xiphinema index* has a high economic impact in vineyards worldwide, by transmitting in the
 42 soil, from plant to plant, the *Grapevine fanleaf virus* (GFLV)³⁻⁵. GFLV, the most severe disease
 43 of grapevines, is responsible for fanleaf degeneration⁶ which occurs in temperate regions of
 44 vine cultivation^{7,8}. *Xiphinema index* is an ectoparasitic^{7,8} nematode with a limited host range and
 45 grapevine is by far its major host⁹. Another major ectoparasitic nematode of grapevine is *X.*

1 *diversicaudatum*, the vector of *Arabidopsis Mosaic virus* (*ArMV*), also involved in grapevine
2 degeneration.

3 The ban of chemical nematicides in an increasing number of countries, due to their adverse
4 environmental effects, has rendered urgent the development of alternative control methods. In
5 grapevine, breeding for nematode-resistant rootstocks is a promising alternative for nematode
6 and subsequent virus controls and resistant accessions are being developed¹⁰⁻¹³. Another
7 alternative is the use of plants with an antagonistic effect on *X. index* to be grown in the fallow
8 period between two successive vine crops¹⁴. In order to evaluate reliably of these control
9 approaches, a better knowledge of the nematode genetic variability is requested. This
10 variability depends mainly on the reproduction mode of the parasite¹⁵. In *X. index*, females
11 reproduce by meiotic parthenogenesis¹⁶ but occasional sexual reproduction may also occur
12 even though males are rare¹⁷.

13 Beside the knowledge of its reproductive mode, the native area of *X. index*, where it should
14 display its highest genetic diversity, is also key information for diversity studies. For *X. index*,
15 this information will help to trace its routes of introduction and will provide a better
16 understanding of its dispersal, which is crucial for sustainable management of GFLV disease.
17 Because GFLV might have been introduced in Western vineyards from the Middle-East with
18 domesticated grapevine¹⁸, the native origin of the vector nematode *X. index* in the Eastern
19 Mediterranean Basin has also been evoked¹⁹⁻²³. Nevertheless, there is still no conclusive
20 argument available today to support this assumption.

21 We report hereafter diversity studies conducted in this objective using molecular markers.
22 Mitochondrial genes sequences are maternal markers commonly used as markers for
23 phylogeography studies²⁴. We first studied the mitochondrial genetic diversity of *X. index*, as
24 a meiotic parthenogenetic species, and of the related amphimictic species *X. diversicaudatum*,
25 and thus compared vector species with different mating systems, using the gene coding for
26 *Cytochrome B* (*CytB*). We then focused on *X. index* and described the phylogeographical
27 patterns among a high number of sampling locations worldwide, using *CytB* marker together
28 with three other mitochondrial genes, the *ATP synthase subunit 6* (*ATP6*), the *Cytochrome c*
29 *oxidase subunit 1* (*COI*) and the *NADH dehydrogenase subunit 4* (*ND4*). Finally, we relied on
30 eight microsatellite markers specific for *X. index*²⁵ to complete the new data set with nuclear
31 markers.

32 Our data set for *X. index* contributed to i) evaluate its variability in comparison with the
33 panmictic species *X. diversicaudatum*, ii) establish the relationship between the geographical
34 patterns of genetic variability observed in its current distribution area, iii) define putative
35 nematode dissemination routes from its Eastern Mediterranean native regions and draw
36 hypotheses on the correlative dispersal itineraries of its domesticated grapevine from the very
37 early Antiquity periods, and iv) get a better appraisal of the worldwide nematode genetic
38 diversity for its control. Finally, our data illustrate how the respective genetic variabilities of a
39 pest and its host plant may give back each other key clues to decipher their common
40 dissemination routes.

1 MATERIAL AND METHODS

3 Origin of nematode samples and individuals

4 The two dagger nematodes, *X. index* and *X. diversicaudatum*, have been initially obtained from
5 field soil samples (Table S1). Within a same vineyard plot, a sample corresponded to one soil
6 lump obtained from a single or a few clods dug in a same GFLV (*X. index*) or ArMV (*X.*
7 *diversicaudatum*) focus. The total soil weight ranged from 0.25 to 2.0 kg. All 82 samples
8 containing *X. index* originated from grapevine plots (Table S1). Out of them, 33 originated
9 from greenhouse rearing of a field sample. Rearing was performed in pots on grapevine or fig
10 hosts grown in a greenhouse collection initiated in 1993 at INRA-ISA, Sophia-Antipolis,
11 France (Table S1). Approx. half of the other *X. index* samples originated from soils received
12 by express mail and directly used on their date of reception for nematode extraction and storage
13 at -80°C . The last half of remaining samples consisted in individuals extracted from soil by
14 their provider, fixed in 70 % ethanol solution and sent to INRA-ISA for direct processing or
15 storage at -80°C . Samples of *X. diversicaudatum* were obtained from different field crops
16 (Table S1). They were introduced and either reared at INRA-ISA collection in pots on their
17 original host plant or on a set of other host plants or directly extracted from soil on the date of
18 reception and stored at -80°C . In our study, the 82 samples of *X. index* covered a geographic
19 area ranging from Middle- and Near East, Eastern Mediterranean, to Europe, North and South
20 America, while the 8 samples of *X. diversicaudatum* originated from France, The Netherlands
21 and Italy. In both *Xiphinema* species, 3 individuals per sample were amplified for
22 mitochondrial and microsatellite markers. When several haplotypes or genotypes were detected
23 in the 3 individuals from a same sample, 3 additional individuals, when available, were
24 characterized for both markers types, in order to confirm the results and/or detect putative
25 additional variants. Because of their diverse sampling characteristics and histories and of their
26 low numbers, we always designated in our study the individuals from a same origin as samples
27 and not as populations.

29 Preparation of DNA templates from single individuals

30 DNA from a single nematode individual was isolated by a simplified procedure: an adult or
31 juvenile was hand-picked, placed in 0.5 ml PCR tube containing 50 μl lysis buffer (KCl 50mM,
32 Gelatin 0.05%, Tris pH 8.2 10mM, Tween 20 0.45%, Proteinase K 60 $\mu\text{g}/\text{ml}$ and MgCl_2
33 2.5mM). Then eppendorf tubes were alternatively moved from liquid nitrogen to 55°C water
34 bath for 10 times in order to facilitate break down of the nematode body. This step was followed
35 by an incubation at 60°C for 90 min (tubes were vortexed at least once during incubation to
36 help breaking up tissues) and by a heating at 95°C for 10 min to inhibit the reaction of
37 Proteinase K. Finally, individuals were cooled at 4°C , vortexed briefly (2-3 sec) and centrifuged

1 shortly at 6,000 rpm for 30 sec. DNAs were stored at -20°C until use for PCR or further
2 experiments.

3

4 **Primer design for *CytB*, *ATP6*, *ND4* and *CO1* genes**

5 We performed BLAST alignments between each of the *CytB*, *ND4*, *CO1* and *ATP6* sequences
6 of *X. americanum* obtained by He et al.²⁶ (accession number NC_005928) and the EST
7 database from *X. index* on nemaBLAST (http://nematode.net/NN3_frontpage.cgi). We found
8 putative partial sequences of the four mitochondrial homologous genes in *X. index* (contigs
9 XI01293 for *CytB*, XI01255 for *ATP6*, XI01185 for *ND4* and XI01295 for *CO1*). For each
10 gene, an alignment was performed between the *X. americanum* sequence and each of these
11 partial sequences using Clustal W build in Mega software version 6.0. Then, specific primers
12 were designed from these alignments, for each gene, and used to amplify specific fragments
13 from *X. index* (*CytB*-852 bp, *ND4*-644 bp, *CO1*-998 bp and *ATP6*-550 bp) and *X.*
14 *diversicaudatum* (*CytB*-559 bp) (Table S2).

15

16 **PCR amplification, purification and sequencing of mitochondrial genes**

17 The primers designed enabled the amplification of partial sequences of all four mitochondrial
18 genes from *X. index*, and of *CytB* gene from *X. diversicaudatum*. All amplifications were
19 carried out in 50µL reaction mixture containing 3µL of DNA template, 5 µL of reaction buffer,
20 2 µL of each primer at 10 µM and 0.4 units of Taq polymerase (AmpliTaq, Applied
21 Biosystems/Perkin Elmer). Amplifications were performed on a Hybaid thermocycler with the
22 following steps: (i) 95°C for 3 min; (ii) 35 cycles with 30 sec at 94°C, 1 min at 59°C (52°C for
23 *ATP6*) and 1 min 30 sec at 72°C; and (iii) ending with 72°C for 5 min. PCR products were run
24 on 1% agarose gel in 0.5X TAE. When the samples gave unspecific bands, 40µL of PCR
25 product was loaded into 1.5% agarose gels in TAE and the band was recovered from the gel
26 with a sterile scalpel and purified using the MinElute Gel Extraction Kit protocol (Qiagen).
27 DNA sequences obtained from purified PCR products were aligned visually. Because all the
28 sequences are protein coding and have no introns or gaps, alignment was straightforward.

29

30 **Microsatellite genotyping**

31 We used a set of 8 out of the 9 primer pairs (Table S3) designed by Villate et al.²⁷. In our study,
32 we used forward primers carrying directly the fluorescent tag instead of the additional
33 fluorescent M13 tail. PCR reactions were carried out in 10 µL simplex reaction containing 2
34 µL of DNA extract, 2 µL of 5x Taq reaction buffer, 0.15 units of Taq polymerase, 5U/µL Taq
35 DNA polymerase (AmpliTaq, Applied Biosystems/Perkin Elmer), 0.3 µL of each primer
36 (forward primers were fluoro-labelled with a FAM, PET, NED or VIC dye at the 5' end) at 10

1 μ M. For each reaction we mixed 4 pairs of primers in one tube called mix 1 (microsatellites
2 Xi29, Xi04, Xi16 and Xi13) or mix 2 (microsatellites Xi24, Xi22, Xi32 and Xi27) (Table S3).
3 Reactions were carried out in a PTC-100 thermocycler (MJ Research) with the following
4 amplification conditions: 95°C for 3 min; 30 cycles with 30 sec at 94°C, 1 min 30 sec at 57°C
5 and 1 min 30 sec at 72°C; and ending with 72°C for 10 min. PCR products were stored at -
6 20°C or used directly by adding 1.5 μ L of PCR product into 9.5 μ L of formamide containing
7 0.5 μ L of the internal lane standard marker (500-LIZ). Samples were read on a R3130XL
8 Genetic Analyzer 16 Capillary system (Applied Biosystems).

9 10 **Data analysis**

11 For mitochondrial genes, we computed the mean distances between sequences with the Mega
12 software version 6.0²⁸ using a Kimura-2-Parameters model and the Maximum Likelihood (ML)
13 method to build phylogenetic trees^{29,30}. The sequence of *X. americanum* was included as an
14 outgroup in our phylogenetic analysis²⁶. For microsatellite data, we first analyzed the results
15 with the GeneMarker program version 1.75 (Applied Biosystems). Then we used the
16 POPULATION software version 1.2.31 (available at <http://bioinformatics.org/populations/>) to
17 compute the distance between individuals by Cavalli-Sforza and Edwards distance (CSE)³¹ to
18 draw Neighbor-Joining trees.

19 20 **RESULTS**

21 22 **Mitochondrial diversity in *X. index* and *X. diversicaudatum* with *CytB* gene**

23 The *CytB* tree grouped in one clade all samples of *X. index* and in another clade all samples of
24 *X. diversicaudatum* (Fig. S1 A). Thus, each species is gathered in a monophyletic group. Then
25 we estimated the evolutionary intra- and interspecific divergences between *CytB* sequences
26 using a representative individual of all mitochondrial haplotypes (see Table S4 for values of all
27 10 haplotypes including the 6 haplotypes obtained from *CytB* sequences and 4 additional
28 haplotypes obtained hereafter from concatenated mitochondrial sequences). A very low
29 divergence (0.0 to 0.012) was obtained among *X. index* haplotypes while divergence was much
30 higher among *X. diversicaudatum* haplotypes (0.018 to 0.074). A high interspecific divergence
31 among *X. index*, *X. diversicaudatum* and *X. americanum* was revealed (0.336 to 0.357 between
32 *X. index* and *X. diversicaudatum*, 0.909 to 0.915 between *X. index* and *X. americanum* and
33 0.897 to 0.937 between *X. diversicaudatum* and *X. americanum*) (Table S4). We then generated
34 a simpler tree than in Fig. S1 using a subset of 35 *X. index* samples and all *X. diversicaudatum*
35 samples (Fig. 1). This subset of *X. index* samples contained 43 individuals comprising one
36 representative individual from each *CytB* mitochondrial haplotype and from each of the 4
37 additional haplotypes obtained hereafter from concatenated mitochondrial sequences. For the

1 predominant mitochondrial haplotypes, it also included, individuals representative of distant
2 sample locations in order to cover as much as possible the geographical distribution area of *X.*
3 *index* (last column of Table S1). In this tree, *X. index* and *X. diversicaudatum* individuals
4 confirmed that they belong to a same monophyletic group with 98% and 100% bootstrap values
5 for each species, respectively (Fig. 1). The *X. index* samples were distributed into several
6 clades.

8 **Phylogeographical pattern in *X. index* inferred from single and concatenated** 9 **sequences of mitochondrial genes**

10 We then still considered the subset of 43 *X. index* representative individuals from 35 samples
11 to amplify the sequences of *ATP6*, *COI* and *ND4* mitochondrial genes. The ML trees (Fig. S2
12 A, B and C), designed for each gene, displayed the same general topology as for *CytB*. Several
13 new haplotypes were obtained for *ATP6* (e.g. in the Tabriz and Sharekord Iranian samples and
14 in the Alasehir Turkish sample) (Fig. S2A) and for *COI* (i.e. the particular haplotype common
15 to South American samples and to some samples from the South Iberian Peninsula and France)
16 (Table 1) (Fig. S2B). By contrast to *CytB* (6 haplotypes with 13 substitutions in the 852-bp
17 sequence, i.e. a polymorphism rate of 1.53%), the *ATP6* sequence was the most polymorphic
18 by displaying a total of 9 haplotypes (variable sites frequency: 1.82%; 10 substitutions in the
19 550-bp sequence), preceding *COI* with 5 haplotypes (variable sites frequency: 1.50%; 15
20 substitutions in the 998-bp sequence) and *ND4* with 4 haplotypes (variable sites frequency:
21 1.40%; 9 substitutions in the 644-bp sequence).

22 In order to reinforce these congruent topologies, a consensus ML tree was generated using
23 the 3044-bp fragment obtained from the concatenated sequences of the four genes (Fig. 2A).
24 *Xiphinema index* individuals were distributed into two major clades, each clade being separated
25 into two subclades (Fig. 2A and Table 1). In the first clade, the first subclade, designated
26 ‘Western’ (W), gathered all individuals from Western Europe, North and South America, while
27 the second subclade, designated ‘Near- and Middle-East’ (NME), grouped individuals
28 encompassing, west to east, Crete, Cyprus, Turkey, Egypt and Iran. In the second clade, the
29 first subclade, designated ‘Israel and Palestine’ (IP), grouped the 3 individuals (3 samples)
30 from Israel and Cis-Jordan that belong to a single haplotype whereas the second subclade,
31 designated ‘Samos and South Italy’ (SSI), grouped 3 individuals (3 samples) displaying two
32 haplotypes.

33 The Western subclade can be divided into two groups, designated Western 1 (W1) and
34 Western 2 (W2), that diverged by a single substitution (*COI*-438) (Table 1). W1 comprised 14
35 individuals (from 14 samples) with a same haplotype ranging from the island of Crete and Pecs
36 in Hungary, in its east geographic expansion, to the island of Madeira and Northern California,
37 in its western expansion. By contrast, W2 comprised 12 individuals (from 12 samples) located
38 in three regions: the South Iberian Peninsula (Spain and Portugal), Bordeaux (France) and

1 Provence (France). The 11 individuals (9 samples) from NME displayed 5 total haplotypes.
2 NME revealed 2 specific haplotypes in the sample Tabriz (Iran) and a single specific haplotype
3 in each of the samples Sharekord (Iran) and Alasehir (Turkey) (Fig. 2A and Table 1).

4 Subclades NME, IP and SSI, from the Central and Eastern Mediterranean, total 8
5 haplotypes while the widely spread subclade W only totals 2 haplotypes (Fig. 2A and Table 1).
6 These data show that the samples from the Central and Eastern Mediterranean are highly more
7 variable than those from Western countries.

9 **Diversity of *X. index* inferred from microsatellite markers**

10 We then considered the 35 samples from which the subset of 43 *X. index* representative
11 individuals had been retained and characterized their multilocus genotypes (MLGs) (3 to 6
12 individuals per sample). While mitochondrial genes had exhibited 10 total haplotypes,
13 microsatellite data revealed 36 MLGs in these samples (Table S5). The microsatellite diversity
14 was thus higher than the mitochondrial diversity. The unrooted microsatellite tree obtained
15 from this subset had very low bootstrap values and this topology suggests a noticeable level of
16 homoplasy in the data (Fig. 3). Nevertheless, microsatellites allowed revealing a better
17 resolution within the subclades obtained from mitochondrial markers (Fig. 3), which is
18 coherent with their higher mutation rate.

19 Several limiting factors and in particular the few nematode individuals available per
20 sample and the high heterogeneity of sampling procedures between samples prevented us to
21 perform population genetics statistics such as comparison of allelic and genotypic richness.
22 Nevertheless, we performed a raw evaluation of the microsatellite diversity of our data. We
23 first analyzed the samples from the Western subclade (W) that were distributed into only 2
24 mitochondrial haplotypes (groups W1 and W2 differing by a single substitution). Within the
25 individuals from the widely spread haplotype W1 (samples mainly from Europe and North
26 America), microsatellite polymorphism was very limited and we designated them as i) the
27 poorly polymorphic subgroup (W1-PP). The individuals from the haplotype W2 ranged into
28 two contrasting microsatellite subgroups: ii) the polymorphic samples (W2-P) that we detected
29 in three regions (South Iberian Peninsula, Bordeaux and Provence), and iii) the samples from
30 Chile and Argentina (W2-CA) corresponding to a single MLG. The W1-PP subgroup (24
31 individuals corresponding to 23 samples) only displayed 5 variant genotypes of which 3 were
32 monophyletic and differed by a single mutation from the dominant genotype (Fig. 3) and 2
33 were highly different (5 and 6 mutations) (Table S5). These latter genotypes were distantly
34 located from the cluster of predominant and poorly variable genotypes (Fig. 3). By contrast,
35 the W2-P subgroup (14 individuals corresponding to 10 samples) was highly diverse by
36 displaying 13 total MLG of which 11 were monophyletic (10 MLGs) and three were
37 paraphyletic.

1 Analysis of the diversity of the NME subclade (11 individuals from 9 samples displaying
2 9 total MLGs in) showed that its individuals were widely dispersed in the tree with the 4 most
3 extreme variant individuals belonging to the Turkish sample Alasehir (1) and to the Iranian
4 samples (3). The four samples of subclade IP were gathered in sister groups, as expected from
5 their geographical proximity. By contrast, the three samples from subclade SSI were split into
6 several clusters that were not sister groups (Fig. 3 and Table S5).

8 DISCUSSION

10 Genetic diversity on *CytB* gene in *X. index* and *X. diversicaudatum*

11 The 98 and 100% bootstrap values supporting nodes separating the two species of *Xiphinema*
12 considered here illustrated that *CytB* is an efficient marker for their specific identification. The
13 nematode *X. diversicaudatum* displays a higher intraspecific genetic divergence than *X. index*.
14 This is an expected result as the two species differ by their reproductive mode which is
15 amphimictic in *X. diversicaudatum* and mainly meiotic parthenogenetic in *X. index*, even
16 though this latter species is able to reproduce occasionally through a sexual mode¹⁷.

18 Genetic diversity of all mitochondrial sequences in *X. index*

19 This study reports four mitochondrial genes partially sequenced, including the *ATP6* gene that
20 was sequenced here for the first time from *X. index*. It shows that the mitochondrial DNA
21 diversity obtained from these sequences within *X. index* samples is very low (from 1.40% in
22 *ND4* to 1.82% in *ATP6*), which confirms previous data obtained with the *CO1* gene³². Similar
23 data have been reported for the parthenogenetic plant parasitic nematodes *Meloidogyne* spp.³³.
24 Nevertheless, those values are much lower than in the amphimictic species *G. pallida* which
25 displayed 12% *CytB* divergence among native Peruvian clades associated to relatively high
26 levels of diversity and gene flow³⁴.

28 The phylogeography of *X. index* is closely linked to grapevine

29 Grapevine is the major host⁹ of *X. index*. Given the ectoparasitic status of the nematode, its
30 long-distance dispersal is linked to grapevine dissemination by man through the transport of
31 rooted plants with their soil substrate¹⁷. This rooted-grapevine ‘dependence’ of *X. index* can
32 make it a reliable marker of the dispersion of its host crop and our results may bring interesting
33 data when faced, in particular, to the grapevine history^{35–37}. The low mutation rate of the *X.*
34 *index* mitochondrial genome and the reduced number of haplotypes highlight how the
35 geographic distribution of nematode genetic variability may support, confirm or even reveal
36 some grapevine dissemination routes.

1 The few archaeological and historical facts available on the origin of grape cultivation are
2 the seeds of domesticated grapes dated from 6000 BCE found in Georgia and in Turkey and
3 the correlative earliest evidence of wine production has been found in the northern Zagros
4 mountains in North-Western Iran at about 5400-5000 BCE³⁸⁻⁴⁰. The Middle-East grape-
5 growing and wine-making area was presumably located in Northern Mesopotamia and may
6 have also encompassed Turkish mountains from eastern Taurus⁴¹. Grape cultivars should have
7 been transplanted into the Central and Southern Zagros Mountains (3000 BCE) on the
8 Mesopotamian east side and the Jordan Valley until Egypt (circa 4000-3000 BCE) on the
9 Mesopotamian west side⁴¹. More recent data on grapevine nuclear microsatellite diversity fit
10 with these putative ancestral domestication events occurring in the Caucasus and the Fertile
11 Crescent⁴².

13 **The mitochondrial phylogeography suggests that the Near- and Middle-East is** 14 **the native area of *X. index***

15 In Europe, *X. index* has never been found in native forests or climax vegetation⁴³. By contrast,
16 in the Middle East, its presence has been reported in Iranian natural woodland (Sturhan in
17 Weischer⁴⁴) where wild grapevines may be common, e.g. in the forests lying along the Caspian
18 Sea⁴⁵. This explains why *X. index* occurs frequently in cultivated grapevines of Northern and
19 Western Iran¹⁹. In our results, the highest mitochondrial diversity is found in the Near- and
20 Middle-East subclade: in particular, the evidence of three different and unique haplotypes in
21 Tabriz and Sharekord Iranian samples suggested this region as being the closest to or
22 corresponding to the native area of *X. index*. Microsatellites, by stating that the NME subclade
23 also displays a high diversity, provide data in agreement with the hypothesis that this area is
24 the cradle of *X. index* prior to have been dispersed by man through grapevine domestication.

26 **Dissemination of Eastern samples from the Near- & Middle-East subclade**

27 In the hypothesis of an expansion of cultivated grapevine from Southern Caucasus regions
28 (including today's North-West Iran and East Turkey), it appears credible that *X. index* has been
29 spread with its grapevine host by two different routes: a first route in Western direction that
30 may have generated the subclade NME (and from which subclade W has then emerged) and a
31 second route in the Southern direction (maybe through Southern Mesopotamia) that gave birth
32 to subclades IP and SSI (Fig. 2B). Later grapevine historical data^{40,46,47} and chloroplast DNA
33 and microsatellite information^{41,42} were used to build the hypothesis that diffusion of viticulture
34 from the Near East occurred through two different routes around the Mediterranean Basin: a
35 Northern way from Eastern to Western Europe (Hittite, Phrygian, Greek and Roman people in
36 the Antiquity⁴⁰) and a Southern way through Egypt, the Maghreb up-to Gibraltar and the

1 Iberian Peninsula (Phoenicians and then Romans in the Antiquity and Arabs from the 7th
2 century ACE).

3 In the Northern route, domesticated grapevines appeared in Minor Asia, Southern Greece,
4 Crete and Cyprus in 3000-2500 BCE and in the Southern Balkans⁴⁸ in 1500-2000 BCE. They
5 finally reached Southern Italy, Southern France, Spain and Portugal in the first millennium
6 BCE⁴⁹. For *X. index*, subclade NME matches the area of the Greek civilization with samples
7 from Western Turkey, Cyprus, Crete and Egypt that might be a representative area of the North
8 Mediterranean route of grapevine varieties. Nevertheless, the detection of an Egyptian sample
9 that marks the Southern part of this putative Greek dissemination area suggests an introduction
10 of the nematode there during the late Antiquity, i.e. during the reign of Alexander the Great or
11 the subsequent Hellenistic period (356-31 BCE) and even later when Egypt has been integrated
12 into the Roman Empire.

13

14 **Dissemination of Eastern samples from IP and SSI subclades**

15 With the grapevine putative historical expansion in mind, we may hypothesize that the samples
16 from Israel and Palestine (subclade IP) are initial steps of the Southern route. Nevertheless, an
17 overlapping of the subclades NME and IP has certainly occurred, at least in Lebanon. This is
18 supported by the fact that, within the *CytB* haplotype obtained from single individuals
19 recovered in two Lebanese locations (recorded in Table S1 but not used in our results for their
20 limitation to a single gene), the first one (Xi-Le-Le; from non specified origin) had the specific
21 IP haplotype while the second one (Xi-Le-Ke; from Kefraya) belonged to the most common
22 NME haplotype (e.g. the same as Xi-Cy-Cy).

23 Because they are closely related to this IP subclade, samples from Samos and South Italy
24 (subclade SSI) might originate from the same region, as Lebanon might have been another
25 dispersion center for *X. index* (Fig. 2B). The fact that, in microsatellite data (Fig. 3), MLGs Xi-
26 Gr-Sa-A and Xi-Gr-Sa-B from Samos on the one hand and from Xi-It-Pa and Xi-It-PC from
27 Southern Italy on the other hand do not belong to sister groups argues for an ancient divergence
28 between them (associated to homoplasy of the markers). In the Antiquity (2000-500 BCE), the
29 Lebanon area was under the authority of the Phoenician kingdom that had also settled many
30 trade harbors in the Southern Mediterranean coast as far as the Southern Iberian Peninsula
31 (Malaga, Cadiz and Lisbon)⁴⁰. Phoenicians have had a great influence in the spread of vine
32 across the Mediterranean Basin⁴⁰ but their possession of Northern Mediterranean trade harbors
33 is weakly plausible. Consequently, nematode introduction from the SSI subclade may have
34 been performed by the Greeks and/or Phoenicians through trade or else in the last two millennia
35 (1500-200 BCE). It may also have occurred later during the Roman Empire epoch.

36

37 **Origin and dissemination of nematodes of the Western subclade**

1 Western samples of *X. index* are closely related to the NME subclade and we hypothesize that
2 they have originated from it. One hypothesis is that *X. index* has been introduced by the Romans
3 after the final unification of Mediterranean territories into the Roman Empire. An alternative
4 hypothesis is that the Greeks have even introduced *X. index* earlier by their distant trading in
5 Northern Mediterranean harbors until Southern Italy and Southern France (1500-200 BCE)
6 (Fig. 2B). Microsatellite data allow to divide European samples from subclade W into i) the
7 poorly polymorphic widely spread samples (W1-PP) on the one hand and ii) the polymorphic
8 Southern Iberian and French (Provence and Bordeaux) samples (W2-P) on the other hand.
9 Those two groups differ only by a single substitution in their mitochondrial haplotype. Thus,
10 they might originate from the same Near- or Middle-East location and have been carried either
11 independently or together, until an unknown intermediate focus.

13 **Dissemination of nematodes of the Western 2 group (W2-P and W-CA)**

14 The high diversity of MLGs in W2-P samples suggests that repeated introductions from the
15 same mitochondrial haplotype have occurred, during the Roman Empire epoch (100 BCE-200
16 ACE), posteriorly to the unification of Western Mediterranean territories. Fréjus (Provence),
17 an important port and the capital of the new Roman Narbonensis Province in 22 BCE, was a
18 place of quick development of viticulture at that time. Almost simultaneously in Bordeaux,
19 archaeological data show that cultivated grapevine has intensively developed from 50 to 250
20 ACE⁵⁰. The clustering of Fréjus, Bordeaux and South Iberian Peninsula samples (for both
21 mitochondrial and microsatellite data) advocates for dissemination events that occurred in a
22 limited time scale and maybe from a common origin. The long and peaceful Roman period in
23 the Western Mediterranean has been certainly a prerequisite for this wide scale diffusion of the
24 vine together with the nematode.

25 In the Iberian Peninsula (and *a fortiori* in its Southern part), chloroplast and microsatellite
26 diversity^{41,42} support the hypothesis of a much earlier introduction of grapevine, probably by
27 the Phoenicians, making this country a secondary center of domestication with centralization,
28 intermixing and exchange of varieties. If we consider that subclade SSI is linked with the
29 Phoenician grapevine dissemination, our current data do not support the hypothesis of another
30 nematode route of Phoenician origin up-to the Southern Iberian Peninsula. However, it is also
31 actually possible that our data dealing with the nematode do not allow tracking those putative
32 early-introduced Phoenician grapevines into Spain because they were nematode free. This early
33 grapevine introduction in the Southern Iberian Peninsula by the Phoenicians might explain why
34 some grapevine varieties from South Spain are also recovered in France (e.g. the cultivar
35 ‘Palomino fino’ which is identical to the French cultivar ‘Listan’; Thierry Lacombe, INRA,
36 France, pers. com.).

37 In America, cultivated grapevine has been imported from Europe since the 16th
38 century^{47,51} by Christian missionaries. Introduction of *X. index* into the New World countries

1 certainly happened through the Spanish colonization of most regions of South America until
2 Argentina and Chile. The complete monomorphism of these South American samples (W2-
3 CA) (Table S5), that illustrates a single introduction event into one of these countries, does not
4 allow to establish whether it occurred directly from Spain, or indirectly from the Canary Islands
5 or another intermediate region^{51,52}. Interestingly studies of the Chilean and Argentinian
6 grapevine historical cultivars show that their diversity was poor and derived from local crosses
7 mainly between two of them (Muscat of Alexandria and Listán Prieto). This poor local
8 grapevine diversity fits with the lack of nematode polymorphism.

9 10 **Origin and dissemination of the Western 1 group (W1-PP)**

11 The second Western haplotype (W1-PP) is almost monomorphic with 19 out of the 24 total
12 samples from this group displaying an identical genotype that ranges from Hungary and Crete
13 eastward to Western and South-Western Europe and until Madeira and California westward
14 (Table S5). This establishes that this haplotype has been spread very recently and presumably
15 from a geographically limited monomorphic focus. This recent dissemination might be
16 consecutive to the massive replants of the vineyards in a huge part of the worldwide grapevine
17 distribution area after the phylloxera crisis in the 19th century^{53,54}. The invariant genotype of
18 most W1 samples may originate from a location initially contaminated by a single introduction
19 event and that has provided nematode-infected rootstocks for the stepwise replant of highly
20 distant vineyards. Interestingly, W1-PP samples exhibit four variant genotypes of which two
21 are highly different and originate from Apulia (Terlizzi) and North Crete, respectively, and two
22 others are less variable but originate from the same Apulian sample and from Sardinia. Such
23 detection of polymorphic individuals in Southern Greece and Southern Italy is quite in line
24 with the hypothesis of a Greco-Roman origin of a portion or of the totality of the Western
25 subclade and these latter regions might be or contain the focus from where the predominant
26 W1 genotype originated (Fig. 2B). Moreover, the recent historical route that presumably lead
27 to its massive dispersal in close linkage with the phylloxera crisis would need to be deciphered.
28 Surprisingly, our data also state that, contrary to what occurred in South America, *X. index* has
29 not been introduced in California by the Spanish colonization from the 16th century but much
30 later from the 19th-20th centuries with vines grafted on phylloxera-resistant rootstocks that
31 compulsorily originated from Europe. Interestingly several locations from the Southern Iberian
32 Peninsula, Bordeaux and Provence (i.e. having W2-P individuals) also harbored W1-PP
33 individuals, which illustrates their double historical infection. Finally, our data illustrated how
34 the respective genetic diversities of a nematode and its host plant may give back each other key
35 clues to decipher their common dissemination routes.

36 37 **Interest of phylogeographical data for reliable evaluation of control methods** 38 **against *X. index***

1 The ban of nematicides in an increasing number of countries, due to their adverse
 2 environmental effects highlights the need for alternative control methods against *X. index*.
 3 Breeding for nematode-resistant rootstock accessions and selection of antagonistic plants are
 4 the major current research fields. For the durability of the resistance sources, together with the
 5 sustainable efficiency of antagonistic plants, an accurate evaluation of the genetic variability
 6 of the pest is a central issue. Evaluation of these control approaches requires the use of samples
 7 representative of the genetic variability of *X. index*. This is primarily dependent on the part of
 8 the overall genetic diversity carried out by these experimental samples among the total diversity
 9 of the nematode. Here, our aim has been to get this primary set of data and to draw a picture of
 10 the genetic diversity of *X. index* at the global scale. Our results have shown that, in order to
 11 reflect this worldwide diversity, it is indispensable to retain nematode haplotypes/genotypes
 12 from both Western and Eastern locations. Thus, choosing a representative nematode haplotype
 13 from each of i) the Western most common genotype (W1-PP), ii) the Near- and Middle East
 14 subclade, and iii) the Samos and South Italy or Israel and Palestine subclades is recommended.

15

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45

46 Author Contributions Statement

47 D.E., V.C.N. and L.V. designed the study; D.E., V.C.N, L.V., P.C. and C.G.G. collected the
 48 samples; V.C.N. and D.E. conducted the literature review; V.C.N., D.E., C.V.G. and L.V.
 49 analyzed the data; V.C.N. and D.E. wrote the paper with inputs from L.V. and O.P.

1 **Additional information**

2 **Data availability statement**

3 EBI accession numbers of the *CytB*, *ATP6*, *COI*, and *ND4* mitochondrial genes sequences
4 (LT996593 to LT996818) are reported in Table S1.

5 Sequences have been uploaded at the URL: <http://www.ebi.ac.uk/ena/data/view/PRJEB26007>

6 They will be available upon manuscript acceptance.

8 **Competing interests**

9 The authors declare that they have no competing interests.

10

11 **Supplemental data**

12 **Supplemental Tables**

13 **Table S1.** List and characteristics of the *Xiphinema index* (Xi) and *X. diversicaudatum* (Xd)
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30 **Table**

1 **Table 1.** Mitochondrial haplotypes obtained using *CytB*, *ATP6*, *COI* and *ND4* gene sequences
2 within the subset of 35 samples (43 individuals) of *X. index* representing the nematode
3 distribution area.

4

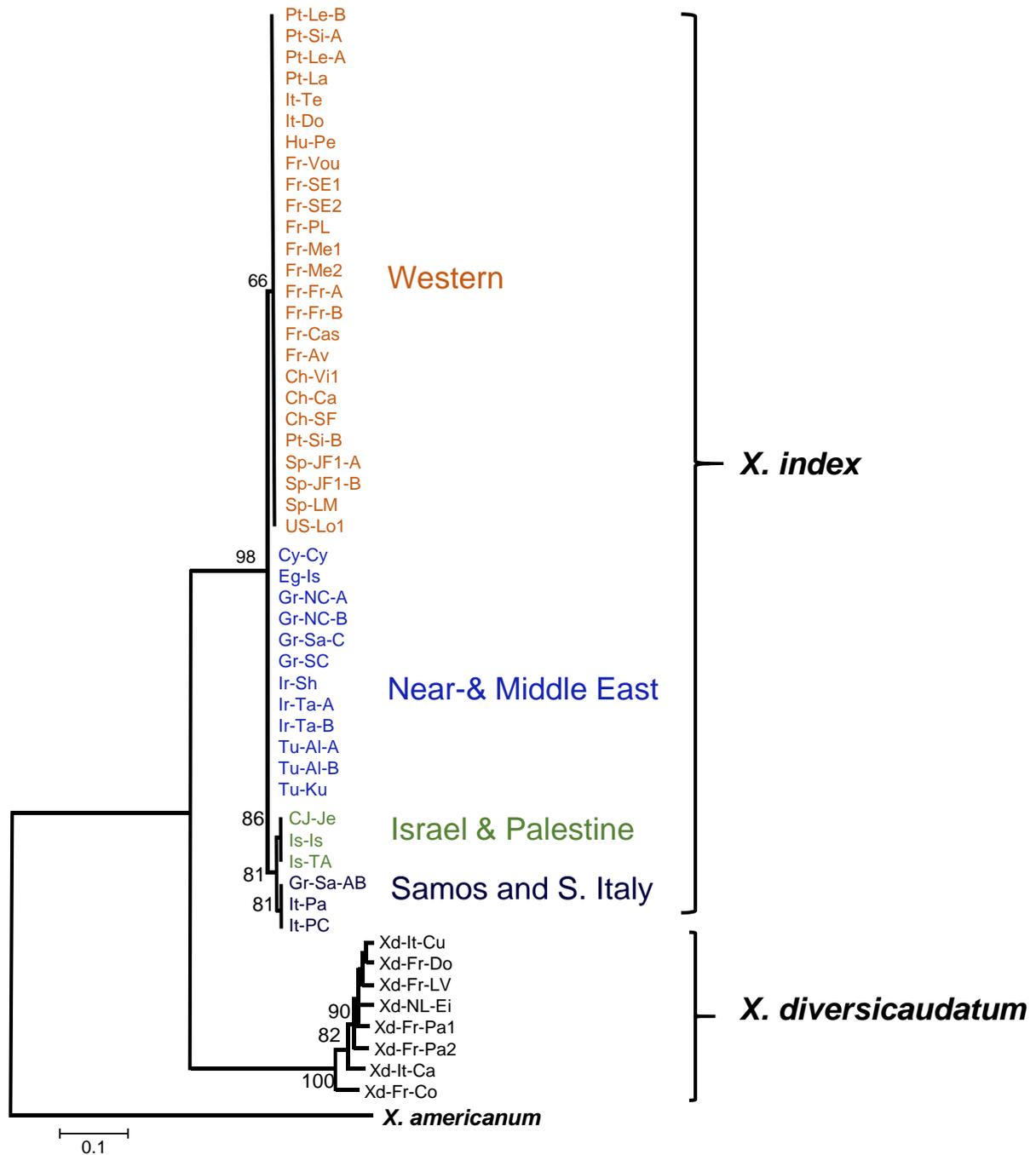
5 **Figures**

6 **Figure 1.** Maximum likelihood phylogenetic tree constructed for *CytB* sequences of *X. index*
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9 **Figure 2.** Phylogenetic tree and correlative hypothesis on the dissemination routes of *X. index*
10 from its native area throughout the Mediterranean basin and beyond.

11 **Figure 3.** Unrooted tree of *X. index* individuals based on microsatellite multilocus genotypes

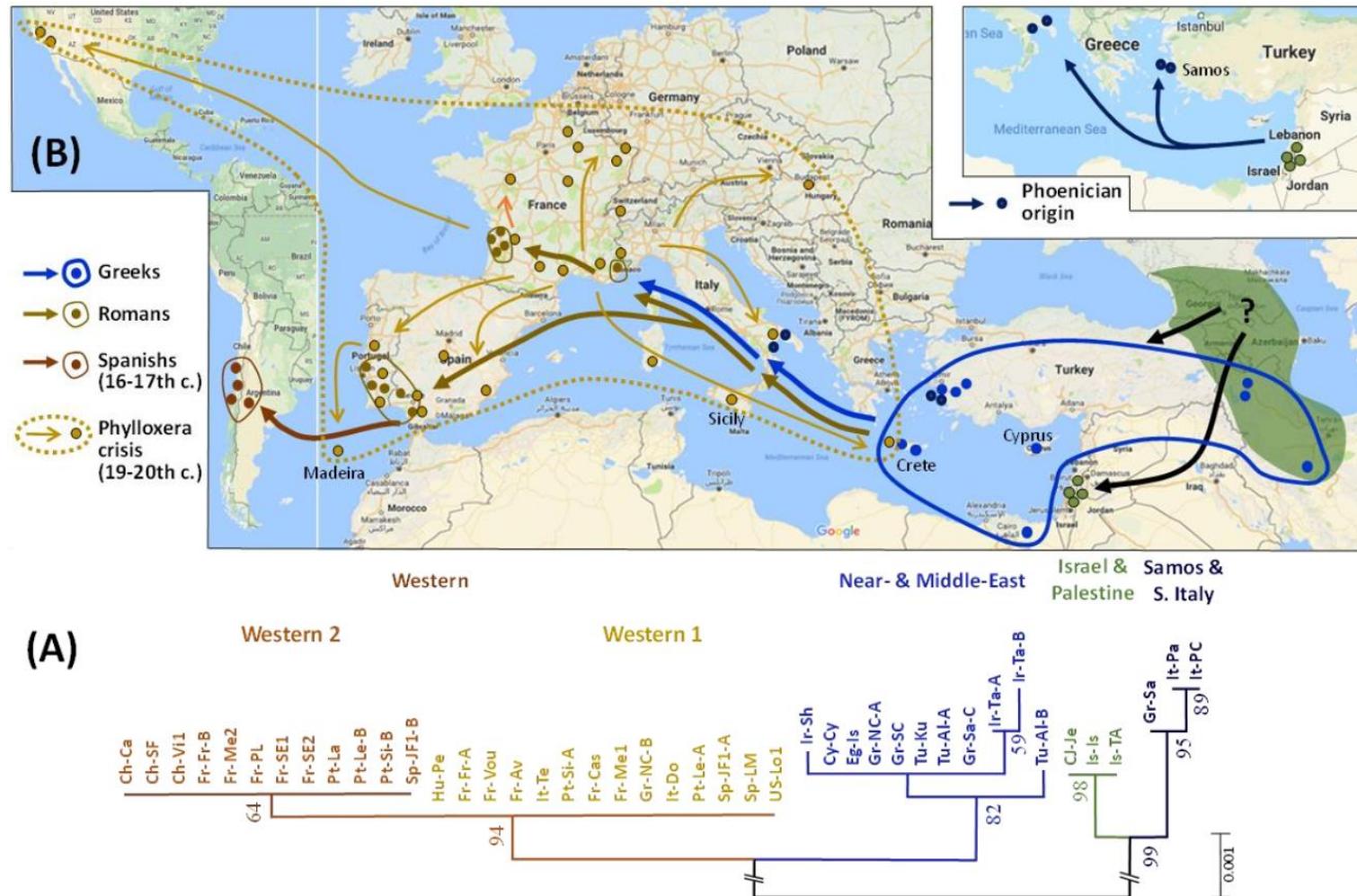
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2

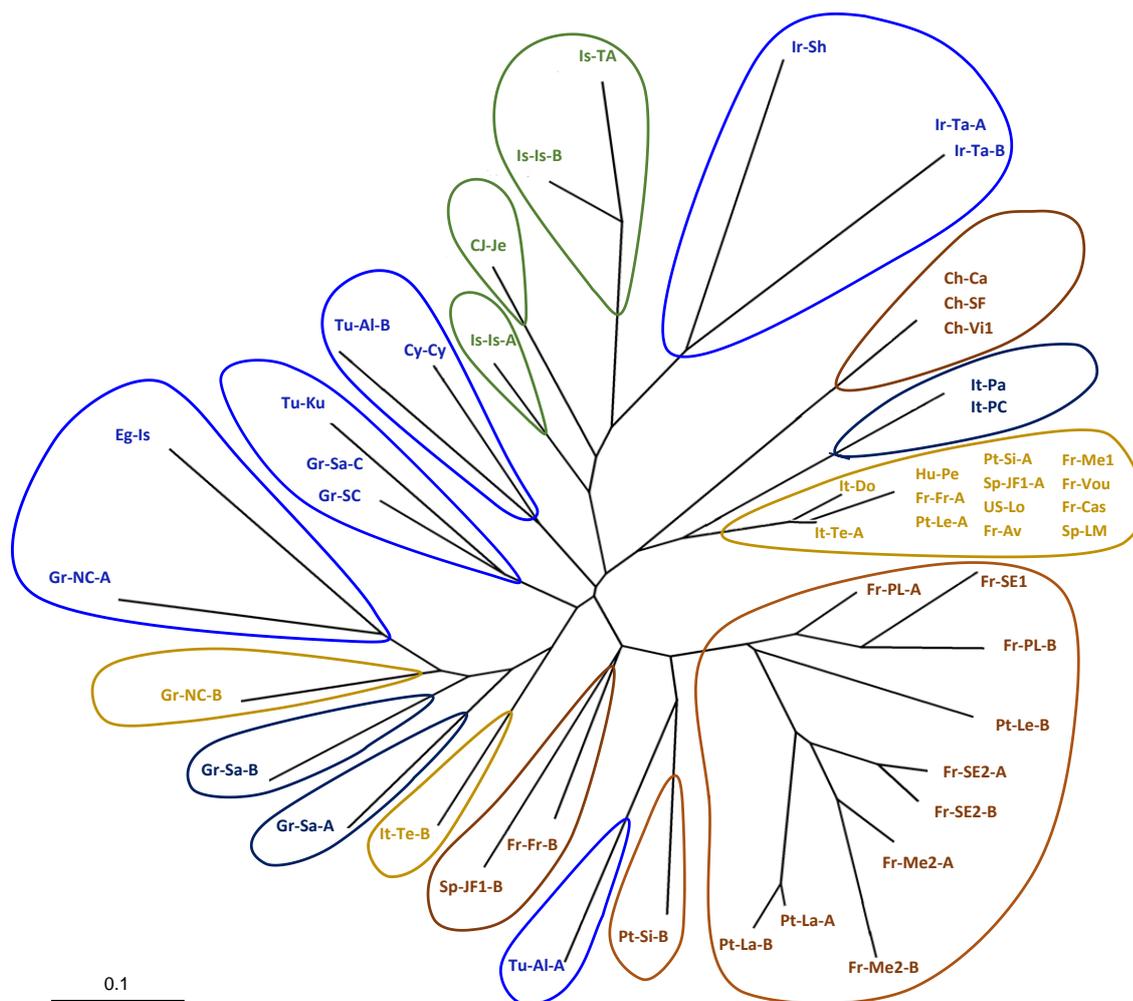
3

4 **Figure 1. Maximum likelihood phylogenetic tree constructed for *CytB* sequences of *X.***
 5 ***index* (subset of 43 representative individuals from 35 samples: see text) and *X.***
 6 ***diversicaudatum* (8 samples) using Mega software version 6.0. *X. americanum* was used**
 7 **as outgroup. Bootstrap values based on 2000 interactions are indicated.**



1

2 **Figure 2. Phylogenetic tree and correlative hypothesis on the dissemination routes of *X. index* from its native area throughout the Mediterranean basin and**
 3 **beyond. (A)** Maximum likelihood (ML) phylogenetic tree for concatenated mitochondrial sequences of *CytB*, *ATP6*, *CO1* and *ND4* from the *X. index* representative
 4 individuals (35 samples). Bootstrap values based on 2000 interactions are indicated. **(B)** Putative dissemination routes inferred from mitochondrial data of the individuals
 5 shown in (A) and from microsatellite data of individuals from the same samples with identical haplotypes but different MLGs. The dark green zone (Transcaucasia and
 6 Southern Caspian Sea regions) encircles the putative native area of domesticated *V. vinifera* grapevine from where *X. index* has been first spread westward (black
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1
 2 **Figure 3. Unrooted tree of *X. index* individuals based on microsatellite multilocus**
 3 **genotypes.** Data were from the subset of 35 samples (see text). This Neighbor-Joining tree
 4 based on distance CSE was performed using POPULATION software version 1.2.32 by 2000
 5 bootstraps. The colors (golden: W1; brown: W2; blue: NME; green: IP and dark blue: SSI)
 6 correspond to the mitochondrial topology (see Table 1). For sample codes, see Table S1.

Supplemental Tables

# individual	# sample	Sample code	Individual code	Country	Region	Location	Host plant	Type	Provider (year)	Accession numbers				# rep. indiv.
										CytB	ATP6	COI	ND4	
1	1	Xi-Fr-SE1	Xi-Fr-SE1	France	Bordeaux	St Emilion	Grape	F	Bordeaux Sciences Agro (2006)	LT996601	LT996690	LT996733	LT996776	1
2	2	Xi-Fr-SE2	Xi-Fr-SE2	France	Bordeaux	St Emilion	Grape	F	Bordeaux Sciences Agro (2006)	LT996602	LT996691	LT996734	LT996777	2
3	3	Xi-Fr-FL	Xi-Fr-FL	France	Bordeaux	Pessac-Leognan	Grape	F	Bordeaux Sciences Agro (2006)	LT996603	LT996692	LT996735	LT996778	3
4	4	Xi-Fr-Me1	Xi-Fr-Me1	France	Bordeaux	Medoc	Grape	F	Bordeaux Sciences Agro (2006)	LT996604	LT996693	LT996736	LT996779	4
5	5	Xi-Fr-Me2	Xi-Fr-Me2	France	Bordeaux	Medoc	Grape	F	Bordeaux Sciences Agro (2006)	LT996605	LT996694	LT996737	LT996780	5
6	6	Xi-Fr-Cah	Xi-Fr-Cah	France	South West France	Cahors	Grape	F	Virginie Viguès, ITV (2007)	LT996606				
7	7	Xi-Fr-Ga	Xi-Fr-Ga	France	South West France	Gaillac	Grape	G	Virginie Viguès, ITV (2007)	LT996607				
8	8	Xi-Fr-VM	Xi-Fr-VM	France	Languedoc	Villeneuve-les-Maguelonne	Grape	G	INRA Sophia (1993)	LT996608				
9	9	Xi-Fr-Cal	Xi-Fr-Cal	France	Cotes du Rhône	Cairame	Grape	G	Marion Claverie, ITV (2012)	LT996609				
10	10	Xi-Fr-CP	Xi-Fr-CP	France	Cotes du Rhône	Chateaufort-du-Pape	Grape	G	Marion Claverie, ITV (2012)	LT996610				
11	11	Xi-Fr-Pb	Xi-Fr-Pb	France	Provence	Ronthevas	Grape	F	Marc Chovelon, ITAB (2012)	LT996611				
12	12	Xi-Fr-Cas	Xi-Fr-Cas	France	Provence	Cassis	Grape	G	INRA Sophia (1993)	LT996612	LT996695	LT996738	LT996781	6
13	13	Xi-Fr-Fr	Xi-Fr-Fr-A	France	Provence	Fréjus	Grape	G	INRA Sophia (1993)	LT996613	LT996696	LT996739	LT996782	7
14	14	Xi-Fr-Fr	Xi-Fr-Fr-B	France	Provence	Fréjus	Grape	G	INRA Sophia (1993)	LT996614	LT996697	LT996740	LT996783	8
15	15	Xi-Fr-Bi	Xi-Fr-Bi	France	Provence	Biot	Grape	F	C. Gutierrez-Gutierrez, CSIC (2010)	LT996615				
16	16	Xi-Fr-SJ	Xi-Fr-SJ	France	Provence	St Jeannet	Grape	F	INRA Sophia (2011)	LT996616				
17	17	Xi-Fr-El	Xi-Fr-El	France	Burgundy	Eiveau	Grape	F	Marion Claverie, ITV (2012)	LT996617				
18	18	Xi-Fr-FM	Xi-Fr-FM	France	Burgundy	Puligny-Montrachet	Grape	F	Marion Claverie, ITV (2012)	LT996618				
19	19	Xi-Fr-Vol	Xi-Fr-Vol	France	Burgundy	Volnay	Grape	F	Marion Claverie, ITV (2012)	LT996619				
20	20	Xi-Fr-Meu1	Xi-Fr-Meu1	France	Burgundy	Meursault	Grape	F	Marion Claverie, ITV (2012)	LT996620				
21	21	Xi-Fr-LS	Xi-Fr-LS	France	Burgundy	Ladoix-Serrigny	Grape	F	Marion Claverie, ITV (2012)	LT996621				
22	22	Xi-Fr-MS	Xi-Fr-MS	France	Burgundy	Morey St Denis	Grape	G	INRA Sophia (1993)	LT996622				
23	23	Xi-Fr-Vou	Xi-Fr-Vou	France	Burgundy	Vougeot	Grape	G	INRA Sophia (1993)	LT996623	LT996698	LT996741	LT996784	9
24	24	Xi-Fr-Wi	Xi-Fr-Wi	France	Alsace	Wintzenheim	Grape	G	INRA Sophia (1993)	LT996624				
25	25	Xi-Fr-Ob	Xi-Fr-Ob	France	Alsace	Obernay	Grape	F	INRA Colmar (2012)	LT996625				
26	26	Xi-Fr-SP	Xi-Fr-SP	France	Alsace	St Pierre	Grape	F	INRA Colmar (2012)	LT996626				
27	27	Xi-Fr-Be	Xi-Fr-Be	France	Alsace	Bergheim	Grape	F	INRA Colmar (2012)	LT996627				
28	28	Xi-Fr-Eg	Xi-Fr-Eg	France	Alsace	Eguisheim	Grape	F	INRA Colmar (2012)	LT996628				
29	29	Xi-Fr-MO1	Xi-Fr-MO1	France	Champagne	Mesnil-sur-Oger	Grape	G	INRA Colmar (2012)	LT996629				
30	30	Xi-Fr-MO2	Xi-Fr-MO2	France	Champagne	Mesnil-sur-Oger	Grape	F	INRA Colmar (2012)	LT996630				
31	30	Xi-Fr-Cr	Xi-Fr-Cr	France	Champagne	Cramant	Grape	F	Géraldine Uhiel, CNV (2012)	LT996631				
32	31	Xi-Fr-Av	Xi-Fr-Av	France	Champagne	Avize	Grape	G	INRA Sophia (1993)	LT996632	LT996699	LT996742	LT996785	10
33	32	Xi-Fr-Li	Xi-Fr-Li	France	Val de Loire	Limeray	Grape	G	INRA Sophia (1993)	LT996633				
34	33	Xi-Sp-Bo	Xi-Sp-Bo	Spain	Bollulus del Condado	Bonares	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996634				
35	34	Xi-Sp-PC	Xi-Sp-PC	Spain	Bollulus del Condado	Palma del Condado	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996635				
36	35	Xi-Sp-JF1	Xi-Sp-JF1-A	Spain	Jerez de la Frontera	Jerez de la Frontera	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996636	LT996700	LT996743	LT996786	11
37	35	Xi-Sp-JF2	Xi-Sp-JF1-B	Spain	Jerez de la Frontera	Jerez de la Frontera	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996637	LT996701	LT996744	LT996787	12
38	36	Xi-Sp-JF2	Xi-Sp-JF2	Spain	Jerez de la Frontera	Jerez de la Frontera	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996638				
39	37	Xi-Sp-Ag	Xi-Sp-Ag	Spain	Montilla Moriles	Aguilar	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996639				
40	38	Xi-Sp-PG	Xi-Sp-PG	Spain	Montilla Moriles	Puente Genil	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996640				
41	39	Xi-Sp-Mo	Xi-Sp-Mo	Spain	Montilla Moriles	Moriles	Grape	F	Carlos Gutierrez-Gutierrez, CSIC (2010)	LT996641				
42	40	Xi-Sp-Mu	Xi-Sp-Mu	Spain	Murcia	Murcia	Grape	F	Pablo Castillo, CSIC (2017)	LT996642				
43	41	Xi-Sp-Ju	Xi-Sp-Ju	Spain	Murcia	Jumilla	Grape	F	Pablo Castillo, CSIC (2017)	LT996643				
44	42	Xi-Sp-LM	Xi-Sp-LM	Spain	La Mancha	La Mancha	Grape	G	Maria Arias, CSIC (2005)	LT996644	LT996702	LT996745	LT996788	13
45	43	Xi-Pt-TV	Xi-Pt-TV	Portugal	Torres Vedras	Torres Vedras	Grape	G	INRA Sophia (1993)	LT996645				
46	44	Xi-Pt-AG	Xi-Pt-AG	Portugal	Lisboa	Aldea Galega	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996646				
47	45	Xi-Pt-Le	Xi-Pt-Le-A	Portugal	Lisboa	Leiria	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996647	LT996703	LT996746	LT996789	14
48	45	Xi-Pt-Le	Xi-Pt-Le-B	Portugal	Lisboa	Leiria	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996648	LT996704	LT996747	LT996790	15
49	46	Xi-Pt-Fi	Xi-Pt-Fi	Portugal	Lisboa	Fcanceira	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996649				
50	47	Xi-Pt-Si	Xi-Pt-Si-A	Portugal	Algarve	Silves	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996650	LT996705	LT996748	LT996791	16
51	47	Xi-Pt-Si	Xi-Pt-Si-B	Portugal	Algarve	Silves	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996651	LT996706	LT996749	LT996792	17
52	48	Xi-Pt-La	Xi-Pt-La	Portugal	Algarve	Lagoa	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996652	LT996707	LT996750	LT996793	18
53	49	Xi-Pt-Ta	Xi-Pt-Ta	Portugal	Algarve	Tavira	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996653				
54	50	Xi-Pt-Ma	Xi-Pt-Ma	Portugal	Madeira	Madeira	Grape	F	Carlos Gutierrez-Gutierrez, UE (2017)	LT996654				
55	51	Xi-Hu-Pe	Xi-Hu-Pe	Hungary	South Hungary	Pecs	Grape	G	Peter Nagy, SZIE (2010)	LT996655	LT996708	LT996751	LT996794	19
56	52	Xi-It-PC	Xi-It-PC	Italy	Apulia	Pallo del Colle	Grape	G	Muro Di Vito, CNR (1993)	LT996656	LT996709	LT996752	LT996795	20
57	53	Xi-It-Pa	Xi-It-Pa	Italy	Apulia	Palagianò	Grape	G	Mauro Di Vito, CNR (1994)	LT996657	LT996710	LT996753	LT996796	21
58	54	Xi-It-Te	Xi-It-Te	Italy	Apulia	Terlizzi	Grape	F	Muro Di Vito, CNR (1995)	LT996658	LT996711	LT996754	LT996797	22
59	55	Xi-It-Do	Xi-It-Do	Italy	Sardinia	Dolianova	Grape	F	Pablo Castillo, CSIC (2017)	LT996659	LT996712	LT996755	LT996798	23
60	56	Xi-It-Pi	Xi-It-Pi	Italy	Sardinia	Pimentel	Grape	F	Pablo Castillo, CSIC (2017)	LT996660				
61	57	Xi-It-CG	Xi-It-CG	Italy	Sicily	Charamonte Gufli	Grape	F	Nicola Greco and Alberto Troccoli, CNR (2017)	LT996661				
62	58	Xi-Gr-Sa	Xi-Gr-Sa-A	Greece	Samos	Samos	Grape	G	Emmanuel Tzortzakakis, NARF (2005)	LT996662	LT996713	LT996756	LT996799	24
63	58	Xi-Gr-Sa	Xi-Gr-Sa-B	Greece	Samos	Samos	Grape	G	Emmanuel Tzortzakakis, NARF (2005)					
64	58	Xi-Gr-Sa	Xi-Gr-Sa-C	Greece	Samos	Samos	Grape	G	Emmanuel Tzortzakakis, NARF (2005)	LT996663	LT996714	LT996757	LT996800	25
65	59	Xi-Gr-SC	Xi-Gr-SC	Greece	Crete	South Crete	Grape	G	Emmanuel Tzortzakakis, NARF (2005)	LT996664	LT996715	LT996758	LT996801	26
66	60	Xi-Gr-NC	Xi-Gr-NC-A	Greece	Crete	North Crete	Grape	G	Emmanuel Tzortzakakis, NARF (2006)	LT996665	LT996716	LT996759	LT996802	27
67	60	Xi-Gr-NC	Xi-Gr-NC-B	Greece	Crete	North Crete	Grape	G	Emmanuel Tzortzakakis, NARF (2006)	LT996666	LT996717	LT996760	LT996803	28
68	61	Xi-Cy-Cy	Xi-Cy-Cy	Cyprus	Cyprus		Grape	G	John Philiis, Ministry of Agriculture (1994)	LT996667	LT996718	LT996761	LT996804	29
69	62	Xi-Le-Le	Xi-Le-Le	Lebanon	Lebanon	Lebanon	Grape	F	Caroline Ojeil, IRAL (2011)	LT996668				
70	63	Xi-Le-Ke	Xi-Le-Ke	Lebanon	Lebanon	Kefraya	Grape	F	Caroline Ojeil, IRAL (2012)	LT996669				
71	64	Xi-Is-Is	Xi-Is-Is	Israel	Israel	Israel	Grape	G	David Nevo, Plant Health Service (1994)	LT996670	LT996719	LT996762	LT996805	30
72	65	Xi-Is-TA	Xi-Is-TA	Israel	Israel	Tel-Aviv	Grape	G	Mshael Mrdecheh, Volcani Center (2005)	LT996671	LT996720	LT996763	LT996806	31
73	66	Xi-CJ-Je	Xi-CJ-Je	Os-Jordan	Os-Jordan	Jericho	Grape	G	Mshael Mrdecheh, Volcani Center (2005)	LT996672	LT996721	LT996764	LT996807	32
74	67	Xi-Ir-Sh	Xi-Ir-Sh	Iran	Bakhtiari province	Shahreکرد	Grape	G	Majid Oia, Univ Shahrekord (2005)	LT996673	LT996722	LT996765	LT996808	33
75	68	Xi-Ir-Ta	Xi-Ir-Ta-A	Iran	Azerbaïdjan province	Tabriz	Grape	G	Sokhandan Bashir, Univ Tabriz (2006)	LT996674	LT996723	LT996766	LT996809	34
76	68	Xi-Ir-Ta	Xi-Ir-Ta-B	Iran	Azerbaïdjan province	Tabriz	Grape	G	Sokhandan Bashir, Univ Tabriz (2006)	LT996675	LT996724	LT996767	LT996810	35
77	69	Xi-Tu-AI	Xi-Tu-AI-A	Turkey	Manisa province	Alasehir	Grape	F	Gaip Kaskavali and Ibrahim Mistanoglu, Ege Univ (2013)	LT996676	LT996725	LT996768	LT996811	36
78	69	Xi-Tu-AI	Xi-Tu-AI-B	Turkey	Manisa province	Alasehir	Grape	F	Gaip Kaskavali and Ibrahim Mistanoglu, Ege Univ (2013)	LT996677	LT996726	LT996769	LT996812	37
79	70	Xi-Tu-Ku	Xi-Tu-Ku	Turkey	Manisa province	Kurmkuyucak	Grape	F	Gaip Kaskavali and Ibrahim Mistanoglu, Ege Univ (2013)	LT996678	LT996727	LT996770	LT996813	38
80	71	Xi-Eg-Is	Xi-Eg-Is	Egypt	Suez Canal	Isrmilla	Grape	F	Mohamed Yousef Banora, Ain Shams University (2017)	LT996679	LT996728	LT996771	LT996814	39
81	72	Xi-Ch-Vi1	Xi-Ch-Vi1	Chile	Center Chile	Vicuna	Grape	G	Enrique Tapia Vera, SAG (2006)	LT996680	LT996729	LT996772	LT996815	40
82	73	Xi-Ch-Vi2	Xi-Ch-Vi2	Chile	Center Chile	Vicuna	Grape	G	Pablo Meza, INIA Santiago (2016)	LT996681				
83	74	Xi-Ch-SF	Xi-Ch-SF	Chile	Center Chile	San Felipe	Grape	G	Enrique Tapia Vera, SAG (2006)	LT996682	LT996730	LT996773	LT996816	41
84	75	Xi-Ch-CT	Xi-Ch-CT	Chile	Center Chile	Calera de Tango	Grape	G	Maria Moreno, SAG (2006)	LT996683				
85	76	Xi-Ch-Ca	Xi-Ch-Ca	Chile	Center Chile	Casablanca	Grape	G	Enrique Tapia Vera, SAG (2006)	LT996684	LT996731	LT996774	LT996817	42
86	77	Xi-Ch-LP	Xi-Ch-LP	Chile	Center Chile	La Pintana	Grape	F	Pablo Meza, INIA Santiago (2017)	LT996685				
87	78	Xi-Ch-IM	Xi-Ch-IM	Chile	Center Chile	Isla de Maipo	Grape	F	Pablo Meza, INIA Santiago (2017)	LT996686				
88	79	Xi-Ch-MP	Xi-Ch-MP	Chile	Center Chile	Monte Patria	Grape	F	Pablo Meza, INIA Santiago (2017)	LT996687				
89	80	Xi-Ar-VS	Xi-Ar-VS	Argentina	Mendoza	Villa seca	Grape	F	Marcelo Doucet, Univ. Cordoba (2018)					
90	81	Xi-US-Na	Xi-US-Na	USA	North California	Napa	Grape	G	Andy Walker, UC Davis (1993)	LT996688				
91	82	Xi-US-Lo	Xi-US-Lo	USA	Central Valley	Lodi	Grape	G	Andy Walker, UC Davis (1993)	LT996689	LT996732	LT996775	LT996818	43
1	1	Xd-Fr-Pa1	Xd-Fr-Pa1	France	Bordeaux	Paulliac	Grape	F	INRA Sophia (2006)	LT996593				
2	2	Xd-Fr-Pa2	Xd-Fr-Pa2	France	Bordeaux	Paulliac	Grape	F	INRA Sophia (2006)	LT996594				
3	3	Xd-Fr-Co	Xd-Fr-Co	France	Cognac	Cognac	Grape	G	INRA Sophia (1994)	LT996595				
4														

Gene	Species	Primer name	Primer sequence (5'-3')	Fragment size	Sequence length
<i>CytB</i>	<i>X. index</i>	XiCytBL1	GATGAGGATTCGGGAGCTTA	939	852
		XiCytBR2	AAGGCTCTTCTACGGTTGG		
	<i>X. diversicaudatum</i>	XdCytBF1	GGGGAGCRACAGTYATTATCAA	559	559
		XdCytBR2	TGAATCTTCAGTTTAATARAGGGTATG		
<i>ND4</i>	<i>X. index</i>	XiND4L	TTGAGGTAACCAACCGGAAC	667	644
		XiND4R	AAGAAGGTGTAGGAGGAATAGAAGAA		
<i>CO1</i>	<i>X. index</i>	XiCO1L	GGTTACAATGCACGCTTTTC	1145	998
		XiCO1R	CGGCGAGGTATACCCTGAAT		
<i>ATP6</i>	<i>X. index</i>	XiATP6-F3	TTTGTTTAACTATTTTGCCCTTTG	550	550
		XiATP6-R3	AATATGAAGAAAACGAAAGCTTGAA		

Table S2. Primers designed to amplify the partial sequences of the mitochondrial genes *CytB*, *ATP6*, *CO1*, and *ND4* in *Xiphinema index* and *X. diversicaudatum*. Fragment size [in base pairs (bp)] is the length of the partial sequence amplified and sequence length (in bp) is the number of nucleotides sequenced.

Locus (GenBank Accession no.)	Primer sequences and fluorescent tag	T _a (°C)	Repeat in sequenced allele	Size range of alleles (bp)
Xi29 (EU678753)	GTGGCAGAACCCCAATTCCT - VIC TTAGTTACTGCCCCATCC	62	(GA)9	124–136
Xi04 (EU678745)	GTGAGCAAACGCAGAAGAGA - 6-FAM CAAGAAACCGATTGAAATTATGG	55	(TG)11	195–197
Xi16 (EU67874)	CGACAGGTGGCAGTTATTGA - VIC CGCAACGAATAAGGGAAGAG	55	(TC)11	131–155
Xi13 (EU678747)	AGGACGTCCTGCTTTTGGT - PET TGCCTAAAATGGAGGGCTTA	55	(CA)11	215–259
Xi24 (EU678751)	GAGAATCGAGCGTTTTCTG - 6-FAM CGCGAGAATCATCTGCCTA	55	(AC)10	227–237
Xi22 (EU678749)	CAAAGTGTTTTGGGCGAGAT - VIC TGTTCTGTAAGGTCGGCACA	50	(AAAC)8	144–168
Xi32 (EU678754)	ATGACCACCAATGACGAA - NED CCGCCGGTATTTCCAGTAT	55	(GTT)7	147–165
Xi27 (EU678752)	CGGTGCACTGGTATAGTTGC - NED TCGCTGTGGTGATGTTCTTC	55	(GT)8	243–249

Table S3. Characteristics of the eight microsatellite loci developed for *X. index*. T_a: optimal annealing temperature

CytB in X. index

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Xi-Fr-SE1	H1									
Xi-Fr-Me1	H2	0.000								
Xi-Ir-Sh	H3	0.001	0.001							
Xi-Eg-Is	H4	0.001	0.001	0.000						
Xi-Ir-Ta-A	H5	0.002	0.002	0.001	0.001					
Xi-Ir-Ta-B	H6	0.002	0.002	0.001	0.001	0.000				
Xi-Tu-Al-B	H7	0.001	0.001	0.000	0.000	0.001	0.001			
Xi-CJ-Je	H8	0.011	0.011	0.009	0.009	0.011	0.011	0.009		
Xi-Gr-Sa	H9	0.011	0.011	0.009	0.009	0.011	0.011	0.009	0.002	
Xi-It-PC	H10	0.012	0.012	0.011	0.011	0.012	0.012	0.011	0.004	0.001

Min: 0 Max: 0.012

ATP6 in X. index

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	
Xi-Fr-SE1	H1	0.000									
Xi-Fr-Me1	H2	0.000	0.000								
Xi-Ir-Sh	H3	0.004	0.004	0.000							
Xi-Eg-Is	H4	0.002	0.002	0.002	0.000						
Xi-Ir-Ta-A	H5	0.004	0.004	0.004	0.002	0.000					
Xi-Ir-Ta-B	H6	0.004	0.004	0.007	0.005	0.004	0.000				
Xi-Tu-Al-B	H7	0.002	0.002	0.005	0.004	0.005	0.002	0.000			
Xi-CJ-Je	H8	0.007	0.007	0.011	0.009	0.011	0.007	0.005	0.000		
Xi-Gr-Sa	H9	0.009	0.009	0.013	0.011	0.013	0.009	0.007	0.005	0.000	
Xi-It-PC	H10	0.007	0.007	0.011	0.009	0.011	0.007	0.005	0.007	0.002	0.000

Min: 0 Max: 0.013

CO1 in X. index

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Xi-Fr-SE1	H1									
Xi-Fr-Me1	H2	0.001								
Xi-Ir-Sh	H3	0.004	0.003							
Xi-Eg-Is	H4	0.004	0.003	0.000						
Xi-Ir-Ta-A	H5	0.004	0.003	0.000	0.000					
Xi-Ir-Ta-B	H6	0.004	0.003	0.000	0.000	0.000				
Xi-Tu-Al-B	H7	0.004	0.003	0.000	0.000	0.000	0.000			
Xi-CJ-Je	H8	0.012	0.011	0.012	0.012	0.012	0.012	0.012		
Xi-Gr-Sa	H9	0.013	0.012	0.013	0.013	0.013	0.013	0.013	0.001	
Xi-It-PC	H10	0.013	0.012	0.013	0.013	0.013	0.013	0.013	0.001	0.000

Min: 0 Max: 0.013

ND4 in *X. index*

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Xi-Fr-SE1	H1									
Xi-Fr-Me1	H2	0.000								
Xi-Ir-Sh	H3	0.002	0.002							
Xi-Eg-Is	H4	0.002	0.002	0.000						
Xi-Ir-Ta-A	H5	0.002	0.002	0.000	0.000					
Xi-Ir-Ta-B	H6	0.002	0.002	0.000	0.000	0.000				
Xi-Tu-Al-B	H7	0.002	0.002	0.000	0.000	0.000	0.000			
Xi-CJ-Je	H8	0.011	0.011	0.013	0.013	0.013	0.013	0.013		
Xi-Gr-Sa	H9	0.011	0.011	0.013	0.013	0.013	0.013	0.013	0.003	
Xi-It-PC	H10	0.011	0.011	0.013	0.013	0.013	0.013	0.013	0.003	0.000

Min: 0 Max: 0.013

All concatenated genes in *X. index*

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	
Xi-Fr-SE1	H1	0.000									
Xi-Fr-Me1	H2	0.000	0.000								
Xi-Ir-Sh	H3	0.003	0.002	0.000							
Xi-Eg-Is	H4	0.002	0.002	0.000	0.000						
Xi-Ir-Ta-A	H5	0.003	0.003	0.001	0.001	0.000					
Xi-Ir-Ta-B	H6	0.003	0.003	0.002	0.001	0.001	0.000				
Xi-Tu-Al-B	H7	0.002	0.002	0.001	0.001	0.001	0.001	0.000			
Xi-CJ-Je	H8	0.011	0.010	0.011	0.011	0.012	0.011	0.010	0.000		
Xi-Gr-Sa	H9	0.011	0.011	0.012	0.012	0.012	0.012	0.011	0.003	0.000	
Xi-It-PC	H10	0.011	0.011	0.012	0.012	0.012	0.012	0.011	0.003	0.001	0.000

Min: 0 Max: 0.012

CytB in *X. diversicaudatum*

	H1	H2	H3	H4	H5	H6	H7	H8
Xd-Fr-Pa1	H1							
Xd-Fr-Pa2	H2	0.029						
Xd-Fr-Co	H3	0.070	0.074					
Xd-Fr-Do	H4	0.018	0.031	0.070				
Xd-Fr-LV	H5	0.029	0.029	0.074	0.024			
Xd-NL-Ei	H6	0.026	0.029	0.066	0.024	0.029		
Xd-It-Cu	H7	0.028	0.031	0.074	0.018	0.024	0.028	
Xd-It-Ca	H8	0.048	0.048	0.070	0.045	0.050	0.043	0.052

Min: 0.018 Max: 0.074

CytB in *X. index*, *X. diversicaudatum* and *X. americanum*

		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18
Xi-Fr-SE1	H1																		
Xi-Fr-Me1	H2	0.000																	
Xi-Ir-Sh	H3	0.002	0.002																
Xi-Eg-Is	H4	0.002	0.002	0.000															
Xi-Ir-Ta-A	H5	0.002	0.002	0.000	0.000														
Xi-Ir-Ta-B	H6	0.002	0.002	0.000	0.000	0.000													
Xi-Tu-Al-B	H7	0.002	0.002	0.000	0.000	0.000	0.000												
Xi-CJ-Je	H8	0.011	0.011	0.009	0.009	0.009	0.009	0.009											
Xi-Gr-Sa	H9	0.011	0.011	0.009	0.009	0.009	0.009	0.009	0.004										
Xi-It-PC	H10	0.011	0.011	0.009	0.009	0.009	0.009	0.009	0.004	0.000									
Xd-Fr-Pa1	H11	0.342	0.342	0.339	0.339	0.339	0.339	0.339	0.339	0.336	0.336								
Xd-Fr-Pa2	H12	0.345	0.345	0.343	0.343	0.343	0.343	0.343	0.342	0.339	0.339	0.030							
Xd-Fr-Co	H13	0.341	0.341	0.339	0.339	0.339	0.339	0.339	0.347	0.348	0.348	0.069	0.073						
Xd-Fr-Do	H14	0.357	0.357	0.355	0.355	0.355	0.355	0.355	0.354	0.351	0.351	0.018	0.032	0.069					
Xd-Fr-LV	H15	0.351	0.351	0.348	0.348	0.348	0.348	0.348	0.348	0.345	0.345	0.030	0.030	0.073	0.024				
Xd-NL-Ei	H16	0.354	0.354	0.351	0.351	0.351	0.351	0.351	0.351	0.348	0.348	0.026	0.030	0.065	0.024	0.030			
Xd-It-Cu	H17	0.345	0.345	0.343	0.343	0.343	0.343	0.343	0.342	0.339	0.339	0.028	0.032	0.073	0.018	0.024	0.028		
Xd-It-Ca	H18	0.348	0.348	0.345	0.345	0.345	0.345	0.345	0.345	0.342	0.342	0.049	0.049	0.069	0.045	0.051	0.043	0.053	
<i>X.americanum</i>	H19	0.911	0.911	0.909	0.909	0.909	0.909	0.909	0.916	0.915	0.915	0.897	0.939	0.886	0.923	0.937	0.911	0.917	0.926

Table S4. Estimates of evolutionary intra- and interspecific divergences between mitochondrial sequences (Kimura-2-Parameters model) of *X. index* and *X. diversicaudatum*. For *X. index*, divergences are calculated for each mitochondrial gene (*CytB*, *ATP6*, *CO1* and *ND4*) and for their concatenated sequence. In this species, individuals used (first column) are representatives of the 10 final haplotypes (H1 to H10) obtained from concatenated genes. For *X. diversicaudatum*, estimates are calculated on *CytB* sequences only. Individuals used (first column) are representative of the 7 haplotypes (H11 to H17) recovered from the 8 geographic samples of this species. H19 corresponds to the haplotype of *X. americanum*. Minimum and maximum values are given under each table.

Subclade Group Subgroup	Sample code	Individual code	List of MLG	Xi29 (GA)	Xi04 (TG)	Xi16 (TC)	Xi13 (CA)	Xi24 (AC)	Xi22 (AAAC)	Xi32 (GTT)	Xi27 (GT)	
Western (W)	Western 1 W1-PP	Fr-Vou	Fr-Vou	1	113 117	177 179	127 135	196 216	124 216	142 145	227	
		Fr-Wi	Fr-Wi	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Mo	Fr-Mo	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Av	Fr-Av	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Li	Fr-Li	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Me1	Fr-Me1	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Ca	Fr-Ca	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Fr-Fr	Fr-Fr-A	113 117	177 179	127 135	196 216	124 216	142 145	227		
		US-Lo	US-Lo	113 117	177 179	127 135	196 216	124 216	142 145	227		
		US-Na	US-Na	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Sp-LM	Sp-LM	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Sp-Ju	Sp-Ju	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Sp-JF1	Sp-JF1-A	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-TV	Pt-TV	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-AG	Pt-AG	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-Le	Pt-Le-A	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-Si	Pt-Si-A	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-Ta	Pt-Ta	113 117	177 179	127 135	196 216	124 216	142 145	227		
		It-Pi	It-Pi	113 117	177 179	127 135	196 216	124 216	142 145	227		
		Pt-Ma	Pt-Ma	113 117	177 179	127 135	196 216	124 216	142 145	227		
	It-Te	It-Te-A	113 117	177 179	127 135	196 216	124 216	142 145	227			
	It-Do	It-Do	113 115	177 179	127 135	196 216	124 216	142 145	227			
	It-Te	It-Te-B	109 117	177 179	127 135	196 216	124 216	139 145	223			
	Gr-NC	Gr-NC-B	113 117	177 179	131 135	196 216	214 216	148 148	139 145	223		
	Western 2 W2-P	Fr-Fr	Fr-Fr-B	7	113 117	177 179	127 135	196 200	216 216	128 148	136 145	223 227
		Fr-PL	Fr-PL-A	8	109 113	177 179	117 127	200 204	216 216		142 145	223
		Fr-PL	Fr-PL-B	9	113 177	179	127	200 204	216 216		142 145	223
		Fr-Me2	Fr-Me2-A	10	109 113	177 179	127	200 204	214 216	128 148	136 139	223 227
		Fr-Me2	Fr-Me2-B	11	109 113	177 179	117 127	200 204	214 216	128 148	136 139	223 227
		Fr-SE1	Fr-SE1	12	109 113	179	127	200 204	212 216		142 145	
		Fr-SE2	Fr-SE2-A	13	113 117	177 179	113 127	200 204	216 216	128 148	136 139	223 227
		Fr-SE2	Fr-SE2-B	14	113 117	177 179	117 127	200 204	216 216	128 148	136 139	223 227
		Sp-JF1	Sp-JF1-B	15	113 117	177 179	127	196 200	214 216	128 148	136 142	221 227
		Pt-Si	Pt-Si-B	16	113 177	179	127	196 204	216 216	128 140	136 142	223
		Pt-La	Pt-La-A	17	113 177	179	127	200 204	214 216	128 148	136 139	223
		Pt-La	Pt-La-B	18	113 177	179	127	200 204	214 216	128 148	136 139	223
Pt-Pi		Pt-Pi	19	109 113	179	127	196 204	216 216	128 148	136 145	223	
Pt-Le		Pt-Le-B	19	109 113	179	127	196 204	216 216	128 148	136 145	223	
Western 2 W2-CA		Ch-VI1	Ch-VI1	20	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227
		Ch-VI2	Ch-VI2	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227	
	Ch-SF	Ch-SF	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ch-CT	Ch-CT	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ch-CA	Ch-CA	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ch-LP	Ch-LP	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ch-IM	Ch-IM	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ch-MP	Ch-MP	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Ar-VS	Ar-VS	109 113	177 179	129 135	196 200	216 216	140 140	139 142	227		
	Near- & Middle East (NME)	Eg-Ik	Eg-Ik	21	109 117	177 179	127 131	196 196	212 214	124 132	139 139	223 231
Cy-Cy		Cy-Cy	22	113 117	177 179	127	196 200	214 214	124 148	139 142	223	
Gr-NC		Gr-NC-A	23	113 117	177 179	127 131	196 200	214 214	124 148	139 142	223	
Tu-Ku		Tu-Ku	24	109 113	177 179	127 131	196 204	208 216	124 148	139 145	223 227	
Gr-SC		Gr-SC	25	113 177	179	127	196 196	214 216	124 148	139 145	227	
Gr-Sa-C		Gr-Sa-C	113 177	179	127	196 196	214 216	124 148	139 145	227		
Ir-Ta		Ir-Ta-A	26	109 113	179	127	196 196	212 216	132 148	135 139	221 223	
Ir-Ta		Ir-Ta-B	109 179	179	127	196 196	212 216	132 148	135 139	221 223		
Ir-Sh		Ir-Sh	27	113 179	179	113 127	196 220	212 216	148 148	139 145	221	
Tu-AL	Tu-AL-A	28	113 117	177 185	113 127	127	204 216	216 124	128 145	223		
Tu-AL	Tu-AL-B	29	113 177	179	113 127	127	196 216	208 216	124 145	223		
Iran & Pakistan (IP)	Is-Is	Is-Is-A	30	113 117	179	127	196 196	208 216	140 140	142 142	221 227	
	Is-Is	Is-Is-B	31	109 113	177 179	127	196 196	208 208	140 140	135 142	221	
	Is-Je	Is-Je	32	109 117	177 179	127	196 196	208 216	124 140	135 142	221 227	
	Is-TA	Is-TA	33	109 113	177 179	127	228 208	208 216	124 140	135 142	221	
Sains and Southern (SS)	Gc-Sa	Gc-Sa-A	34	117 179	179	127 135	196 204	208 216	148 148	145 223	227	
	Gc-Sa	Gc-Sa-B	35	117 179	179	127 135	196 204	214 216	148 148	145 223	227	
	It-Pa	It-Pa	36	113 179	185	127 135	196 204	216 124	148 148	145 221	227	
	It-PC	It-PC	113 179	185	127 135	196 204	216 124	148 148	145 221	227		

Table S5. Genotypes obtained from 8 microsatellite markers within the subset of 35 samples of *X. index* representing the nematode distribution area. For sample codes, see Table S1. Single letters A, B, or C in an individual code indicate the existence of different haplotypes or MLGs within the same sample. W1-PP, poorly-polymorphic subgroup of Western samples; W2-P, polymorphic subgroup of Western samples; W2-CA, Chilean and Argentinian subgroup of Western samples.

Supplemental Figures

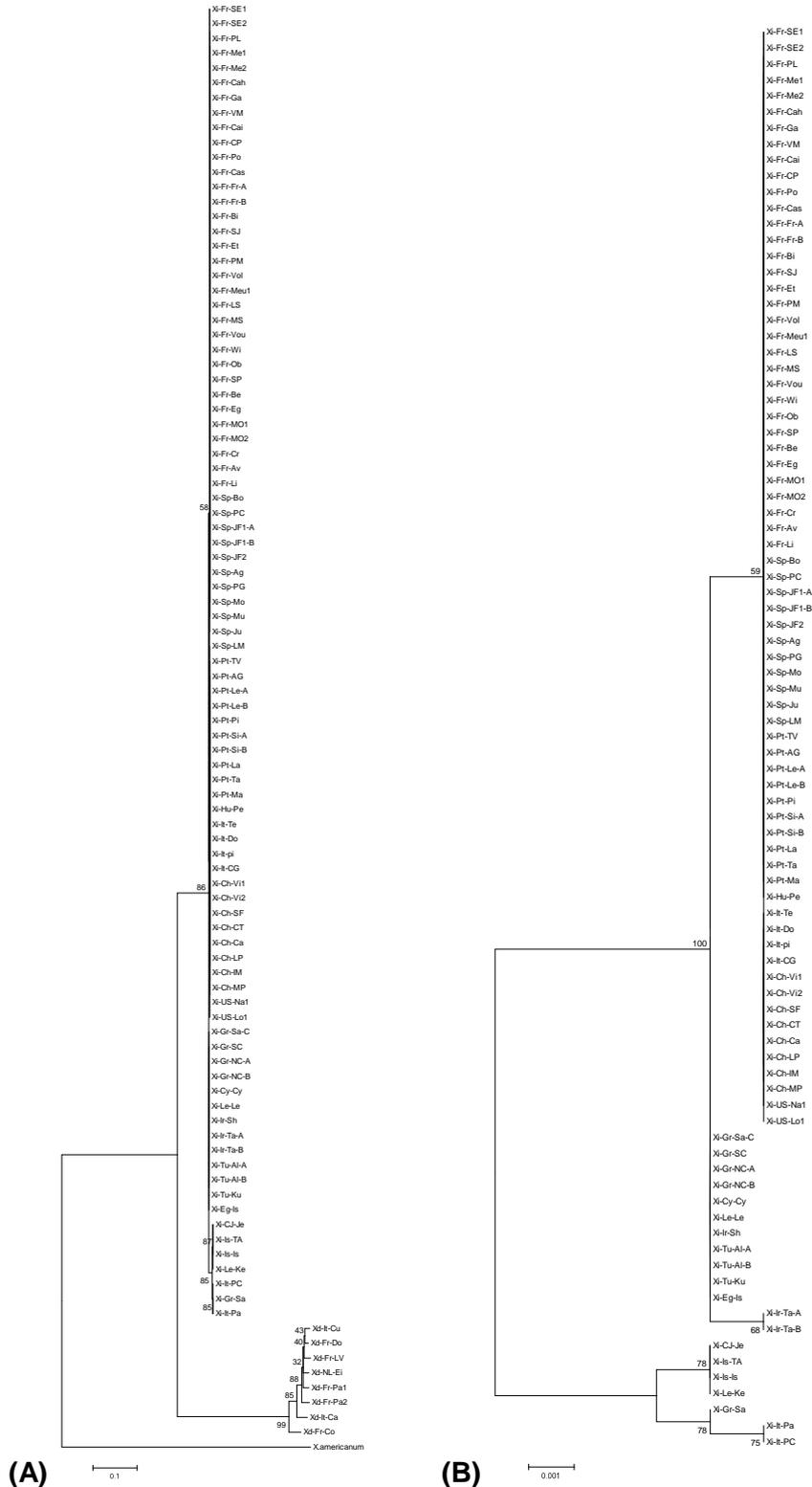
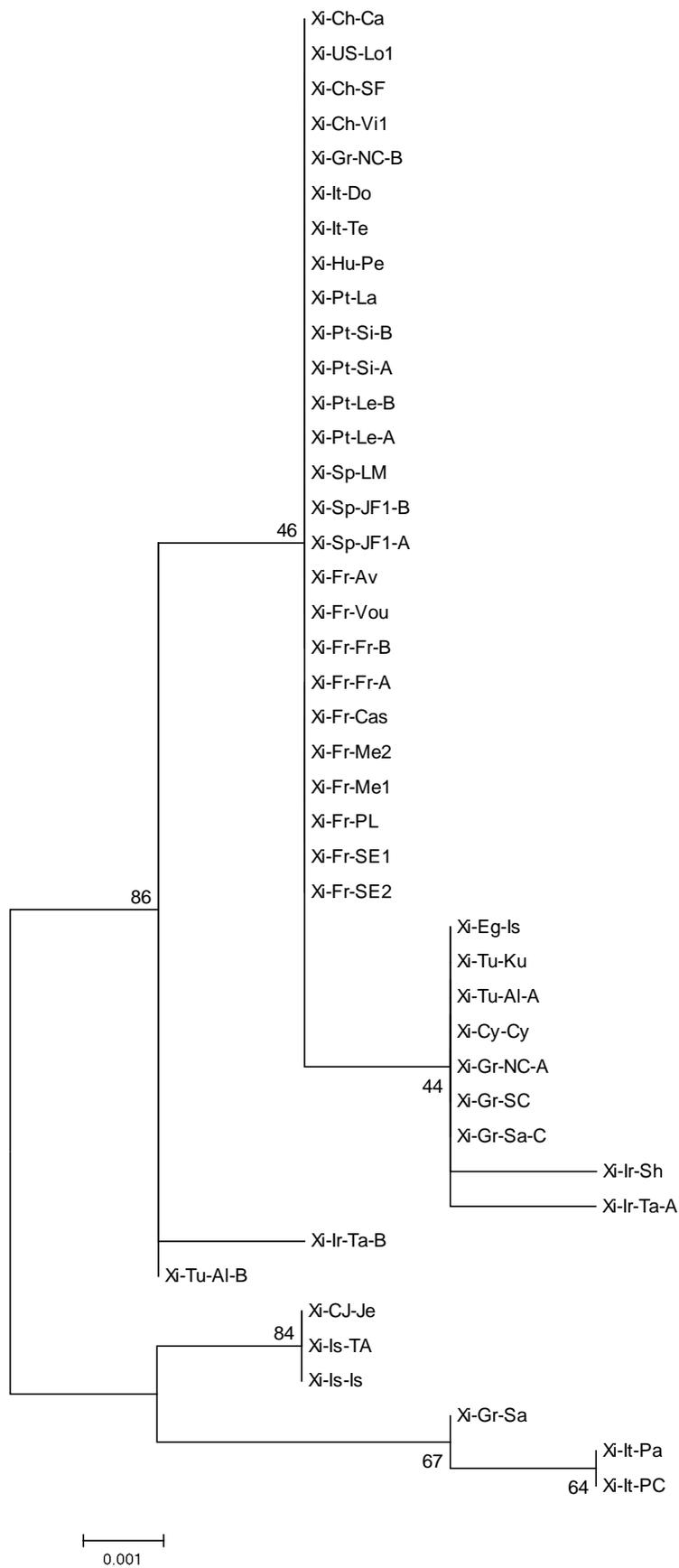
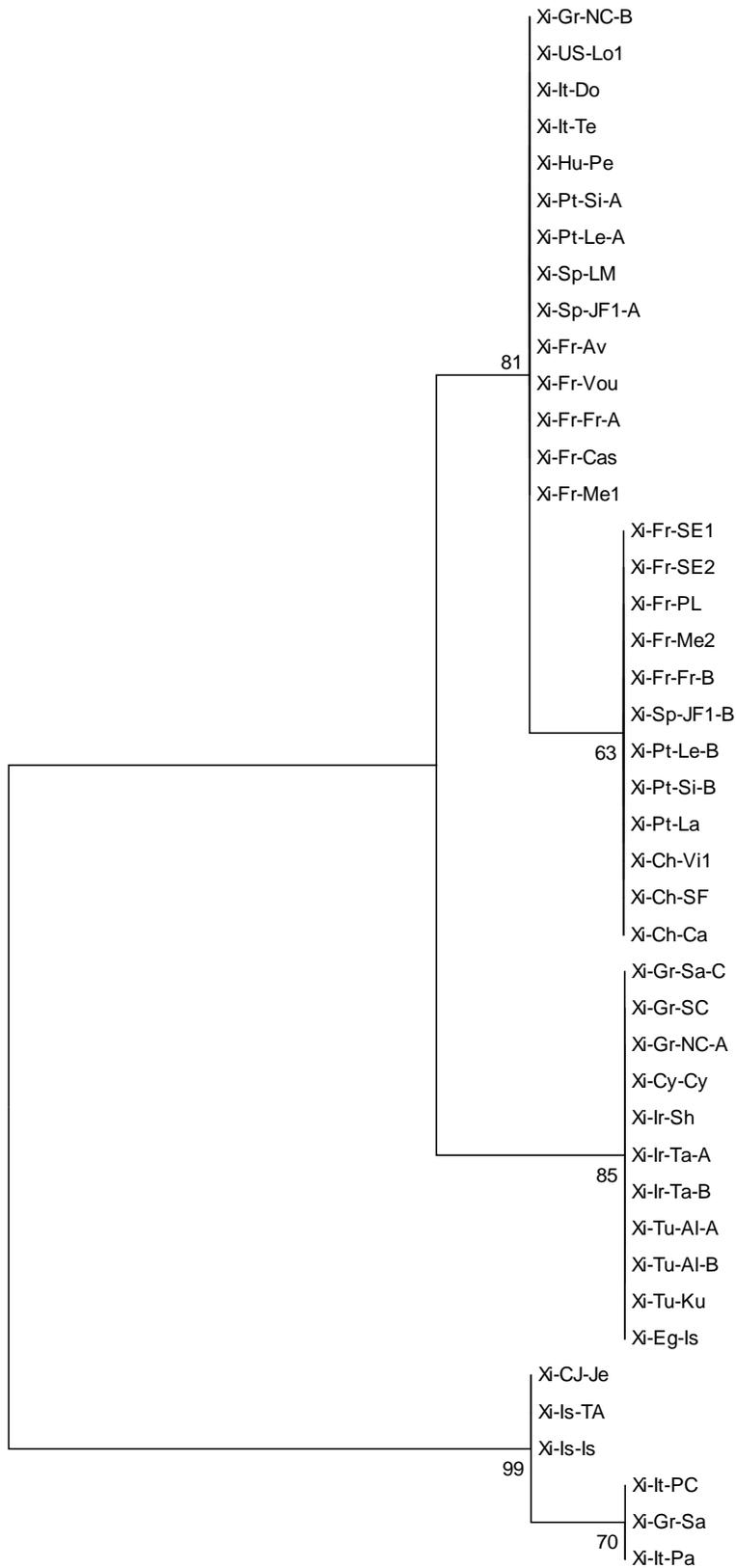


Fig. S1 Maximum likelihood phylogenetic tree constructed for all *CytB* sequences of samples of *X. index* with (A) and without (B) *X. diversicaudatum* (8 individuals). *X. americanum* was used as an outgroup in (A). Bootstrap values based on 2000 interactions are indicated. For codes, see Table S1.

(A) *ATP6*



(B) CO1

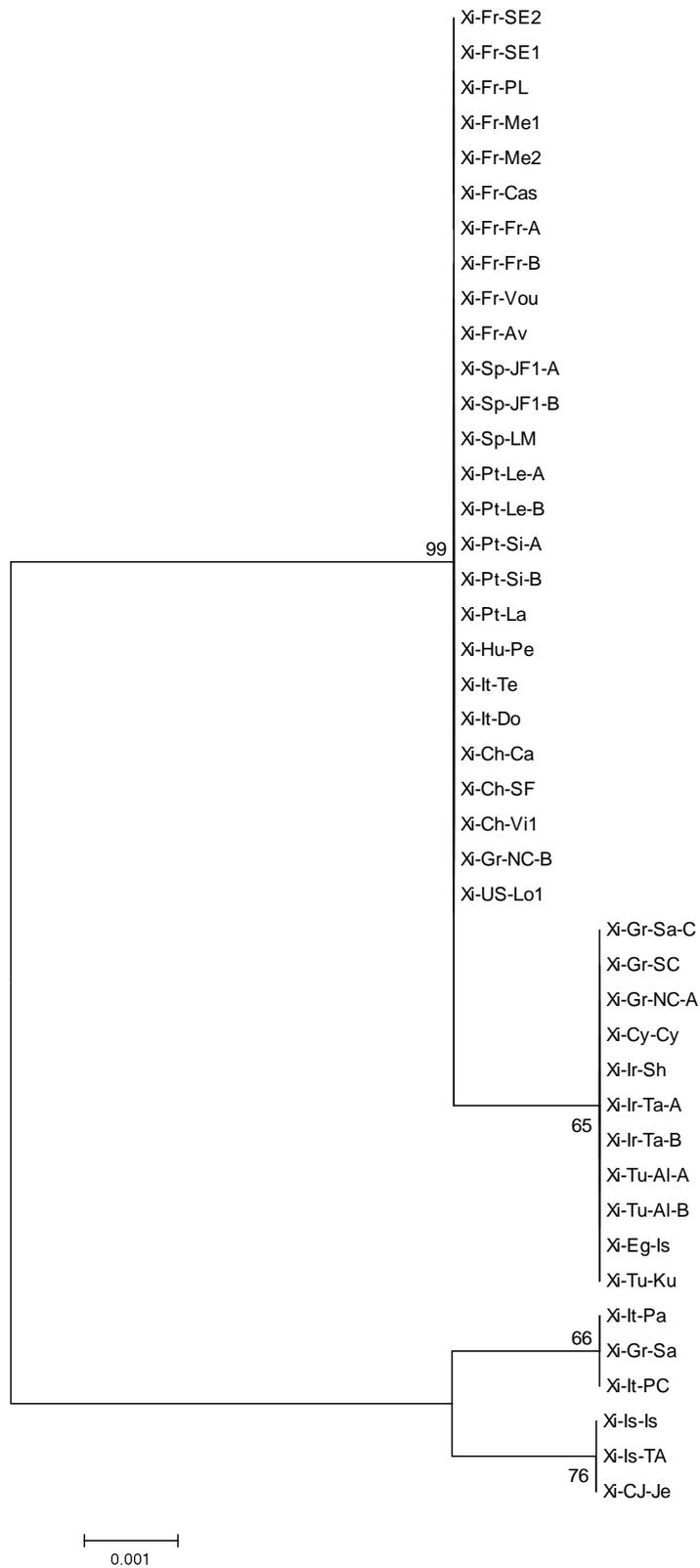
(C) *ND4*

Fig. S2. Maximum likelihood phylogenetic trees for a subset of *X. index* samples based on *ATP6* (A), *CO1* (B) and *ND4* (C). Bootstrap values based on 2000 iterations are indicated. For codes, see Table S1.

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CHAPTER 3

EVALUATION OF THE DURABILITY OF NEMATODE CONTROL STRATEGY BASED ON *MUSCADINIA* DERIVED-RESISTANT ROOTSTOCKS

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CHAPTER 3: EVALUATION OF THE DURABILITY OF NEMATODE CONTROL STRATEGY BASED ON *MUSCADINIA* DERIVED-RESISTANT ROOTSTOCKS

This chapter refers to studies on the mean-term and long-term durability of nematode-resistant rootstocks and cognate nematode adaptation. It develops firstly a resistance durability study conducted over 6 years under controlled conditions. Secondly, it is completed by a field study, established in 1999 in the Domaine du Chapitre (Villeneuve-les-Maguelone) of Montpellier SupAgro by Alain Bouquet. In this latter experiment, GFLV evolution in different rootstock modalities has been monitored by INRA Montpellier, Montpellier SupAgro and ITV, and results reported in this thesis deal with the nematode numbers from a soil sampling performed by ISA in 2015.

III.1. RESISTANCE DURABILITY BASED ON A 6-YEAR ASSESSMENT UNDER CONTROLLED CONDITIONS

This study reports investigations in a 6-year assessment initiated at UMR ISA in 2012. Before this work, studies under controlled conditions had only considered resistance on a duration of one to two years (Bouquet et al., 2000). An Introduction part will summarize the state of the art before my thesis work, i.e. the prerequisite data on i) the nematode material used in the study and ii) the plant material (two types of propagation) used in the study. The subsequent parts dealing with nematode extraction, counting and characterization from 2015 to 2018 (Material and methods, Results...) will refer to the work conducted during the thesis.

III.1.1. Introduction

Studies from Esmenjaud et al. (2010) suggested that, over four years post-inoculation of grapevine obtained from *in vitro*, *X. index* adapts progressively to the resistant accessions (VRH8771 and RPG1). In this previous study, plants originating from *in vitro* were challenged with a single female line (Fréjus, France). This preliminary initial result needs to be confirmed in a more in-depth study. It has been conducted by using plant material obtained from *in vitro* together with classical hardwood cuttings. Moreover, the putative adaptation of the nematode questions the genetic mechanisms that it may set-up: competition between individuals, sexual reproduction... To get a first answer to this question, genetically distant lines have been mixed and their respective evolution has been monitored over several years.

III.1.1.1. Creation and molecular characterization of pure lines of X. index

Creation of lines

A first round of creation of single female descendencies or isofemale lines (referred hereafter as lines or isolates) had been undertaken in the late 1990's among the populations available from the INRA collection at UMR-ISA. This was performed on the sole basis of their geographical distance among the grapevine worldwide distribution area and without any information on their relative diversity. When preliminary information on the worldwide diversity of the populations from this collection has been obtained (Villate, 2008), new populations have been retained for the creation of new lines. Those lines have been created as follows. Young plantlets of fig previously obtained from *in vitro* have been grown in a 100 ml pot for 2-3 months in order to allow the development of a dense root system. For each of the 8 populations retained, 10 adult females have been picked-up and inoculated individually in a 2 cm-deep hole (subsequently carefully filled with fine sand) located 1 cm from the fig plantlet stem within the pot. All plantlets were then placed in trays and grown under controlled conditions in a growth chamber at 25°C and 16L: 8D. Three months after inoculation, plantlet roots were cautiously separated from the soil and observed for the presence of small galls indicative of successful nematode attacks on root tips and of subsequent nematode multiplication. When galling was unambiguously observed, the plantlets and the soil content of their containers were repotted into a 4-liter container to allow better nematode multiplication. Out of the 10 individuals per population (pop.) inoculated singly in pots, a sample of 4 lines (pots) have been retained for further multiplication (Fig. 3.1A).

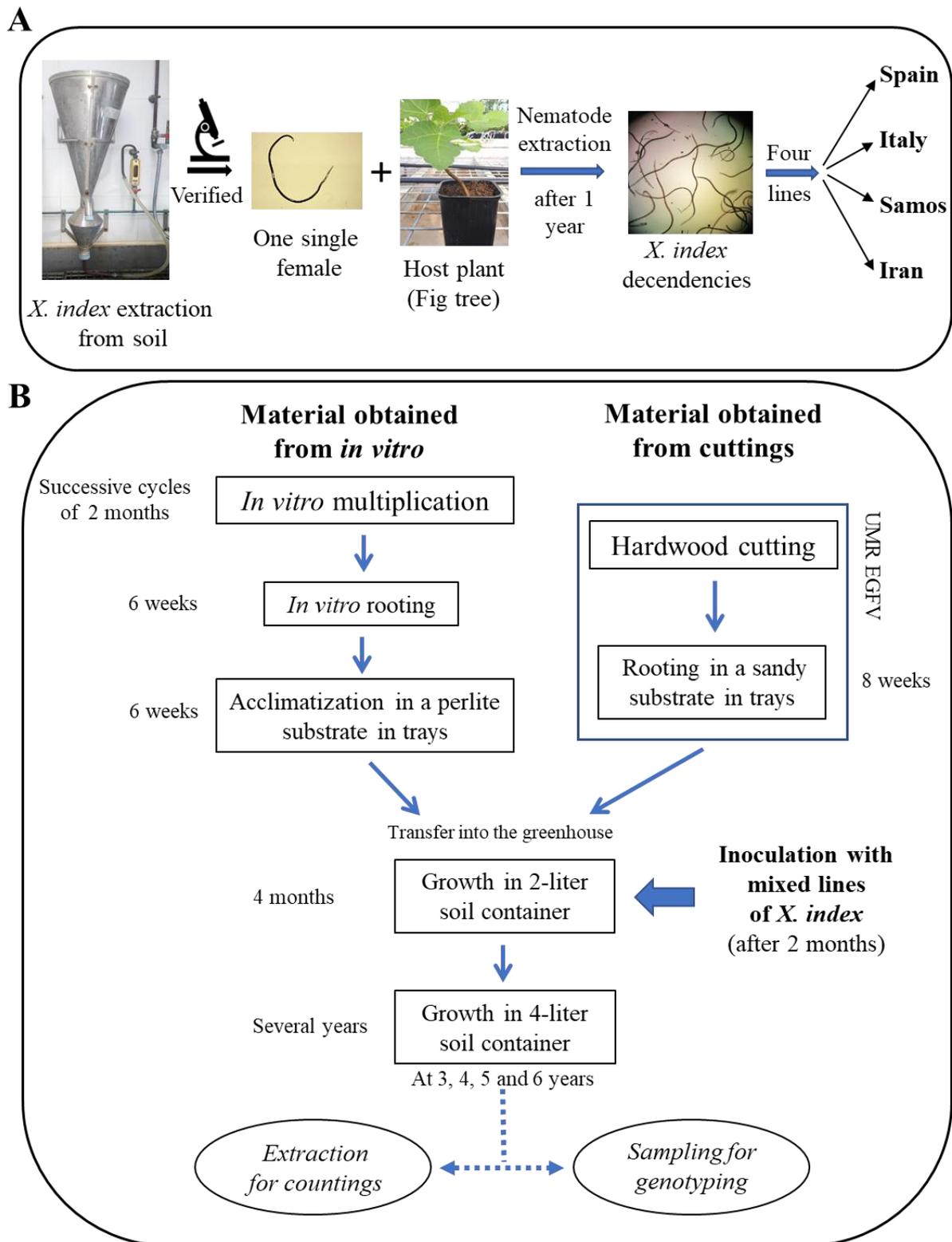


Figure 3.1. Durability study under controlled conditions. (A) Creation of the *X. index* lines from which four ones were retained (Spain, Italy, Samos and Iran). (B) Steps and timing of the study using plants obtained from *in vitro* and from cuttings. At 3, 4, 5 and 6 years, nematodes were extracted and counted; out of them, a sample was genotyped with a set of microsatellite markers.

Molecular characterization of the lines

All new lines (5 lines/pop. x 8 pop. = 40 total lines) were characterized for their mitochondrial haplotype using marker *CytB* and for their microsatellite genotype using the 8 loci available for *X. index* (Table 3.1) (Villate et al., 2009). Purity of lines was verified from a sample of 10 individuals. A single line per population from 4 populations was finally retained and used in the experiments described hereafter. Those 4 final lines were selected because they had either specific alleles or a specific combination of alleles that allowed to identify them and thus to distinguish them unambiguously from each other as shown in Fig. 3.2. Each of the lines retained was then extensively multiplied on a fig host plant in several 4-liter containers. After one more year, nematodes from each line in those pots were used as inoculum for further experiments. The four lines retained originate from (East to West): Iran (Sharekord; Middle East; Xi-Ir-Sh), Greece (Samos; Eastern Mediterranean; Xi-Gr-Sa), Italy (Pallo del Colle; Central Mediterranean; Xi-It-PC) and Spain (La Mancha; Western Mediterranean; Xi-Sp-LM). These lineages belong to three out of the four subclades revealed in our study. In particular, such genetically homogenous nematode material has been used for experiments on the durability of resistance to *X. index* over years (from 2012 to 2018).

Table 3.1. Characteristics of the eight microsatellite loci developed for *X. index* (Villate et al., 2009)

Locus (GenBank Accession no.)	Primer sequences and fluorescent tag	Ta (°C)	Repeat in sequenced allele	Size range of alleles (bp)
Xi29 (EU678753)	GTGGCAGAACCCAATTCCT - VIC TTAGTTACTGCCCCATCC	62	(GA)9	105-117
Xi04 (EU678745)	GTGAGCAAACGCAGAAGAGA - 6-FAM CAAGAAACCGATTGAAATTATGG	55	(TG)11	177-186
Xi16 (EU67874)	CGACAGGTGGCAGTTATTGA - VIC CGCAACGAATAAGGGAAGAG	55	(TC)11	113-136
Xi13 (EU678747)	AGGACGTCCTGCTTTTGGT - PET TGCCTAAAATGGAGGGCTTA	55	(CA)11	197-240
Xi24 (EU678751)	GAGAATCGAGCGTTTTCTCG - 6-FAM CGCGAGAATCATCTGCCTA	55	(AC)10	207-218
Xi22 (EU678749)	CAAAGTGTTTTGGGCGAGAT - VIC TGTTCTGTAAGGTCGGCACA	50	(AAAC)8	124-149
Xi32 (EU678754)	ATGACCACCAATGACGAA - NED CCGCCGGTATTCCAGTAT	55	(GTT)7	128-146
Xi27 (EU678752)	CGGTGCACTGGTATAGTTGC - NED TCGCTGTGGTGATGTTCTTC	55	(GT)8	221-230

Ta: optimal annealing temperature

µsat	Locus 1		Locus 2			Locus 3			Locus 4			Locus 5			Locus 6		Locus 7			Locus 8			
	Xi29 (GA)		Xi04 (TG)			Xi16(TC)			Xi13 (CA)			Xi24(AC)			Xi22(AAC)		Xi32(GTT)			Xi27(GT)			
Allele Line	113	117	177	179	186	113	127	136	197	204	221	207	212	216	124	149	139	142	146	221	223	227	
Spain	113	117	177	179			127	136	197					216	124			142	146			227	
Italy	113			179	186		127	136	197	204				216	124				146	221		227	
Samos		117	177	179			127		197	204		207		216	124					146		223	227
Iran	113			179		113	127		197		221		212			149	139		146	221			

Figure 3.2. Microsatellite characteristics of the four *X. index* lines retained for the study. These lines are designated ‘Spain’ (Xi-Sp-LM), ‘Italy’ (Xi-It-PC), ‘Samos’ (Xi-Gr-Sa) and ‘Iran’ (Xi-Ir-Sh). The multi-locus genotypes, showing the markers or the combination of markers specific for each line, are reported. Red circles highlight the specific alleles (size in bp) for Spain [Xi32 (GTT)-142], Italy [Xi04 (TG)-186], Samos [Xi24 (AC)-207 and Xi27 (GT)-223], and Iran [Xi16 (TC)-113, Xi22 (AAC)-149 ...].

III.1.1.2. The different multiplication modes for plant material

Previous experiments conducted at UMR ISA had used plant material propagated with different methods. Material multiplication had been based on plants obtained either from *in vitro* or from cuttings (Esmenjaud et al., 2010). In this thesis, we have considered and compared a set of accessions propagated alongside with the two modes. We describe hereafter these two propagation methods.

Material obtained from *in vitro*

The plants obtained from *in vitro* originated from *in vitro* cultures first established in the years 2000 at INRA Montpellier from disinfected buds of all appropriate accessions. Standard media derived from Murashige & Skoog (1962) for their introduction (introduction medium) and conservation (multiplication medium). They have been provided by Alain Bouquet to UMR ISA in 2008. At ISA they have been maintained in a dedicated growth room from the UMR facilities (Team M2P2-Modèles et Méthodes pour la Protection des Plantes) at 22°C with a 16-hr photoperiod provided by cool-white fluorescent lamps. Two media, i.e. the multiplication medium (MM) and the rooting medium (RM), also derived from the MS medium (Murashige & Skoog, 1962) (Table 3.2), were used. Every two months, new pieces of shoots have been cut from the old culture, planted for new culture onto the MM medium for conservation purpose (no plant hormones) (Table 3.2). They have then been kept for several rounds as MM cultures until their rooting onto the RM medium (0.5 mg/l IBA = 0.5 ppm IBA) for their subsequent acclimatization and use in the durability experiment (Table 3.2, Figs. 3.1 & 3.3).

Table 3.2. Composition of the media used at UMR ISA for introduction (IM), multiplication (MM) and rooting (RM) of *in vitro* propagation of grapevine plant material

Compound	Introduction medium (IM)	Multiplication medium (MM)	Rooting medium (RM)
Macro Skoog	Diluted 1/2	Standard	Standard
Micro Skoog	1 ml/l	1 ml/l	1 ml/l
Fer-EDTA FeSO ₄ .7H ₂ O	27.8 mg/l	27.8 mg/l	27.8 mg/l
Na ₂ EDTA	37.2 mg/l	37.2 mg/l	37.2 mg/l
Myo-inositol	100 mg/l	100 mg/l	100 mg/l
Nicotinic acid	10 mg/l	1 mg/l	1 mg/l
Thiamine HCl	10 mg/l	30 mg/l	30 mg/l
Pyroxidine	1 mg/l	1 mg/l	1 mg/l
Calcium panthotenate	1 mg/l	1 mg/l	1 mg/l
Biotin	0.01 mg/l	-	-
Casein hydrolysate	100 mg/l	-	-
Glutamin	100 mg/l	-	-
Cysteine chlorhydrate	100 mg/l	1 mg/l	1 mg/l
Phenylalanine	10 mg/l	-	-
Glycocolle	2 mg/l	-	-
Saccharose	20 mg/l	-	-
Sucrose	-	30 g/l	30 g/l
Agar	8 g/l	7 g/l	7 g/l
Indole-3-acetic acid (AIA)	10 mg/l	-	-
Gibberellic acid (GA ₃)	1 mg/l	-	-
Indolebutyric acid (IBA)	-	-	0.5 mg/l
pH adjusted to 5.8 by NaOH			
- none			

After 6 weeks in the RM medium, rooted plantlets were transferred into 50 x 30 x 15 cm trays filled with a perlite substrate without hormones in an acclimatization chamber at 20-22°C for 6 weeks. Plants were then moved to the greenhouse, transplanted individually into 2-liter containers filled with a sterile sand-loam soil and inoculated 2 months later. Two months after inoculation, plants were gently repotted into 4-liter containers. Consequently, the delay between the beginning of the acclimatization of plantlets (end of the *in vitro* steps) and their inoculation has been approx. 3-4 months.

Material obtained from cuttings

Material for the production of rooted cuttings was sampled in spring from dormant grapevine mother plants in the plant repository of INRA EGFV Bordeaux. Hardwood cuttings

comprising two nodes were cut and planted individually for rooting into trays of 150-ml cells filled with a fine-sand substrate. No rooting or shooting hormones were used. Trays were placed onto benches and regularly humidified with a mist system. After 8 weeks, rooted cuttings were transferred to UMR ISA at Sophia-Antipolis and repotted into 2-liter pots filled with a sterile sand-loam soil. Plants were inoculated after 2 months and gently repotted into 4-liter containers after 2 more months (Figs 3.1 & 3.3).

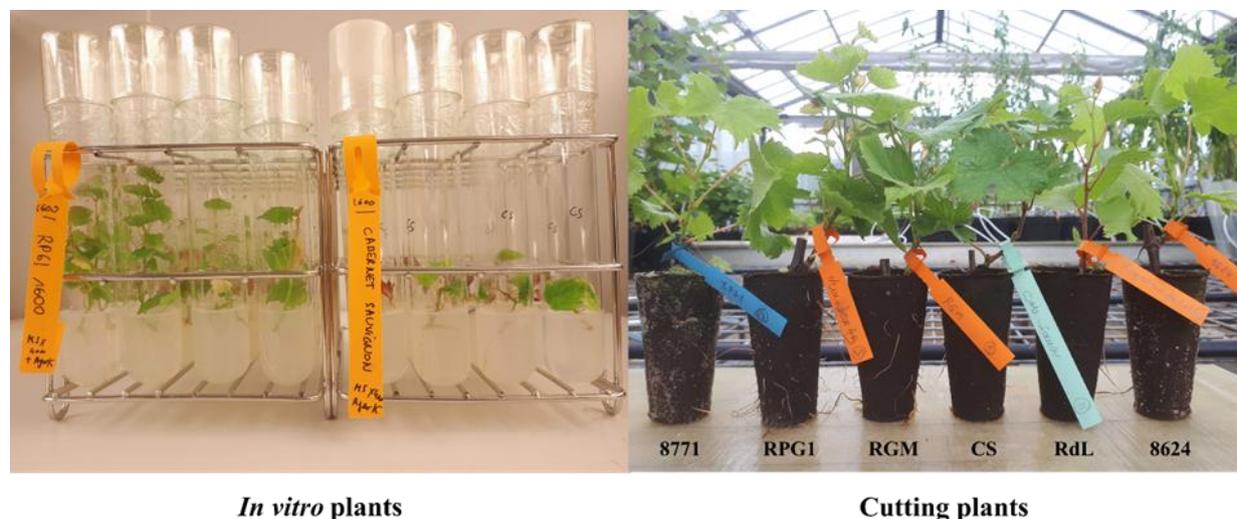


Figure 3.3. Illustration of the initial propagation modes of the plants used for resistance durability experiments. Left: rooted plants in *in vitro* rooting medium; right: rooted plants obtained from cuttings in fine-sand substrate.

III.1.2. Material and methods

III.1.2.1. Materials

Nematode material

As previously described, we used the pure lines (or single-female descendents) from four geographical origins (Figs 3.1 & 3.2), designated Iran (Xi-Ir-Sh), Samos (Xi-Gr-Sa), Italy (Xi-It-PC), and Spain (Xi-Sp-LM).

Plant material

The study used several clonal accessions representing different levels of resistance inherited from the muscadine source NC184-4 (Fig. 1.26): the F1 resistant accession VRH8771 (8771), the BC1 resistant accession Némadex Alain Bouquet (RPG1). The accessions *V. vinifera* cv. Cabernet-Sauvignon (CS) and *V. rupestris* cv. du Lot (RL) were susceptible controls. Two other accessions were used as reference plant material: another susceptible F1 representative accession of the cross *Vitis* x *Muscadinia*, VRH8624 (8624), and the rootstock accession

expressing an intermediate resistance *V. riparia* cv. Gloire de Montpellier (RGM). For each accession, two types of plant material have been evaluated: plants previously rooted as hardwood cuttings ('cutting' material) and plants acclimatized in soil from material previously propagated *in vitro* ('*in vitro*' material). The methods used to obtain *in vitro* and cutting plants are those described above.

III.1.2.2. Methods

We will first describe the full steps of the method for nematode extraction from the soil because it has been used for the inoculation of plant material, for the subsequent evaluation of nematode numbers and, also, to recover the nematodes from the phylogeography study in Chapter 3. We will then report the inoculation method, the experimental design and the microsatellite genotyping protocol used in the greenhouse durability study.

Extraction of nematodes from soil

Nematodes were extracted from soil using the elutriation method adapted from Oostenbrink (Hooper, 1986). Content of each 4-liter pot was divided in two 2-liter soil samples. Each 2-liter sample was processed as follows. Soil was firstly suspended and stirred in 5 liters of water; after 15 seconds of decantation, the suspension was poured onto a sieve with a mesh of 2 mm placed over a 10-liter bucket, and organic material and roots retained by the sieve were eliminated. The resulting 10-liter soil suspension was poured through a 63- μ m sieve. The sieve was rinsed to collect debris and nematodes. Nematodes were then separated from the debris using the modified Oostenbrink elutriator method for large nematodes. For this step, the water flow at the bottom of the funnel was set at 700 ml/min (Fig. 3.4). After their separation in the elutriator, nematodes were retained using again the 63- μ m sieve. The final remains containing nematodes were rinsed with water and placed onto a double cotton wool filter tissue laid on a 1-mm sieve (diameter: 225 mm) in a glazed plate containing 400 ml of water. Active nematodes were recovered from the bottom of the dish after 3 and 7 days. Nematodes (female adults, male adults and juveniles) were counted from the suspension obtained at these two dates under a binocular.

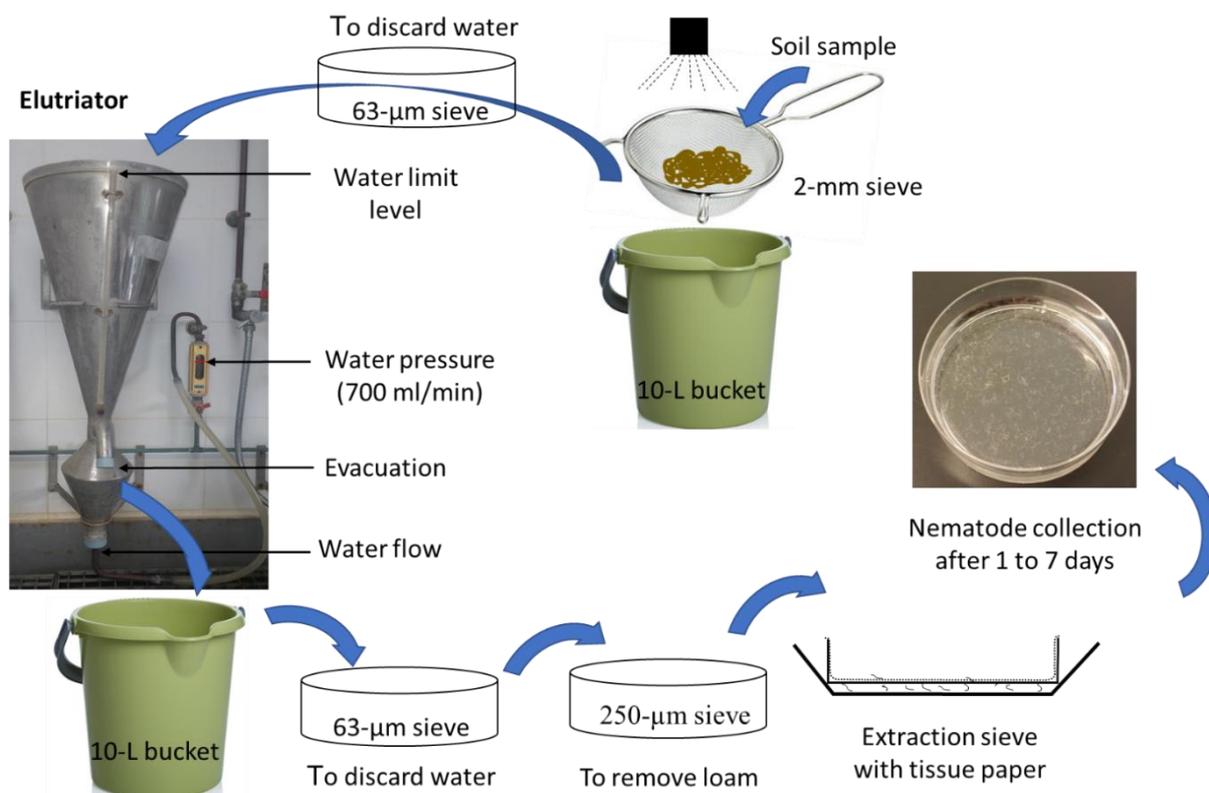


Figure 3.4. Scheme for the extraction of *X. index* nematodes from soil by the Oostenbrink elutriator method.

Inoculation method and experimental design

Mixed inoculations were performed using 200 total individuals comprising 50 individuals from each of the 4 lines. The individuals were suspended in water and deposited into 2 holes, 2-cm deep and 2 cm from the stem. For a same propagation type, pots inoculated with mixed lines were grouped and surrounded by splash screens to avoid cross contaminations with other modalities. Plants of the different accessions were arranged in a randomized design. One or two additional plants from each clone were inoculated with 200 individuals of each line as controls (verification of identity and purity of *X. index* lines). Some additional plants of each clone were used as non-inoculated controls.

In the greenhouse, the protocol with mixed lines has been set up for a total duration of 6 years using the 6 clonal accessions (RPG1, 8771, RGM, CS, RdL and 8624) grown either from cuttings or *in vitro* (Fig. 3.1B). Containers were drip irrigated daily and temperature was regulated to reach a maximum of 30°C. To avoid frost, greenhouse was heated in winter to keep minimal temperatures above 5 °C. Thus, low temperatures permitted winter leaf fall. Every year, after complete leaf fall, plants were pruned by keeping two eyes on each major shoot.

The evaluation of nematode numbers has been performed over 6 years (in 3-, 4-, 5-, and 6-year old plant pots) as reported in Table 4.3. The multiplication of nematodes has been evaluated at each date by extraction of nematodes from all the pot soil content.

Table 3.3. Distribution of the numbers of plants inoculated with mixed lines and used for the adaptation experiment on material aged 3 to 6 years

Age (date)	Plants obtained from <i>in vitro</i>						Plants obtained from cuttings							
	RPG1	8771	RGM	CS	RdL	8624	RPG1	8771	RGM	CS	RdL	8624	Fig	Total
3 years (2015)	5	7	3	4	0	1	6	9	4	3	3	3	0	48
4 years (2016)	7	7	3	5	0	1	10	7	4	4	4	4	1	57
5 years (2017)	7	9	3	5	0	2	10	8	4	4	4	4	1	61
6 years (2018)	7	9	3	5	0	2	11	8	4	4	4	4	2	63
Total	26	32	12	19	0	6	37	32	16	15	15	15	4	229

Microsatellite genotyping

We used a set of 8 out of the 9 primer pairs designed by Villate et al. (2009) (Table 3.1). In our study, we used forward primers carrying directly the fluorescent tag instead of the additional fluorescent M13 tail. PCR reactions were carried out in 10 μ L simplex reaction containing 2 μ L of DNA extract, 2 μ L of 5x *Taq* reaction buffer, 0.15 units of *Taq* polymerase, 5U/ μ L *Taq* DNA polymerase (Ampli*Taq*, Applied Biosystems/Perkin Elmer), 0.3 μ L of each primer (forward primers were fluoro-labelled with a FAM or PET or NED or VIC dye at the 5' end) at 10 μ M. For each reaction we mixed 4 pairs of primers in one tube called mix-1 (microsatellites Xi04, Xi13, Xi16 and Xi29) or mix-2 (microsatellites Xi22, Xi24, Xi27 and Xi32) (Table 3.1). Reactions were carried out in a PTC-100 thermocycler (MJ Research) with the following amplification conditions: 95°C for 3 min; 30 cycles with 30 s at 94°C, 1 min 30 s at 57°C and 1 min 30 s at 72°C; and ending with 72°C for 10 min. PCR products were stored at -20°C or used directly by adding 1.5 μ L of PCR product into 9.5 μ L of formamide containing 0.5 μ L of the internal lane standard marker (500-LIZ). Samples were read on a R3130XL Genetic

Analyzer 16 Capillary system (Applied Biosystems). Fig. 3.5 illustrates the signal of some alleles for the different microsatellite loci and their combinations.

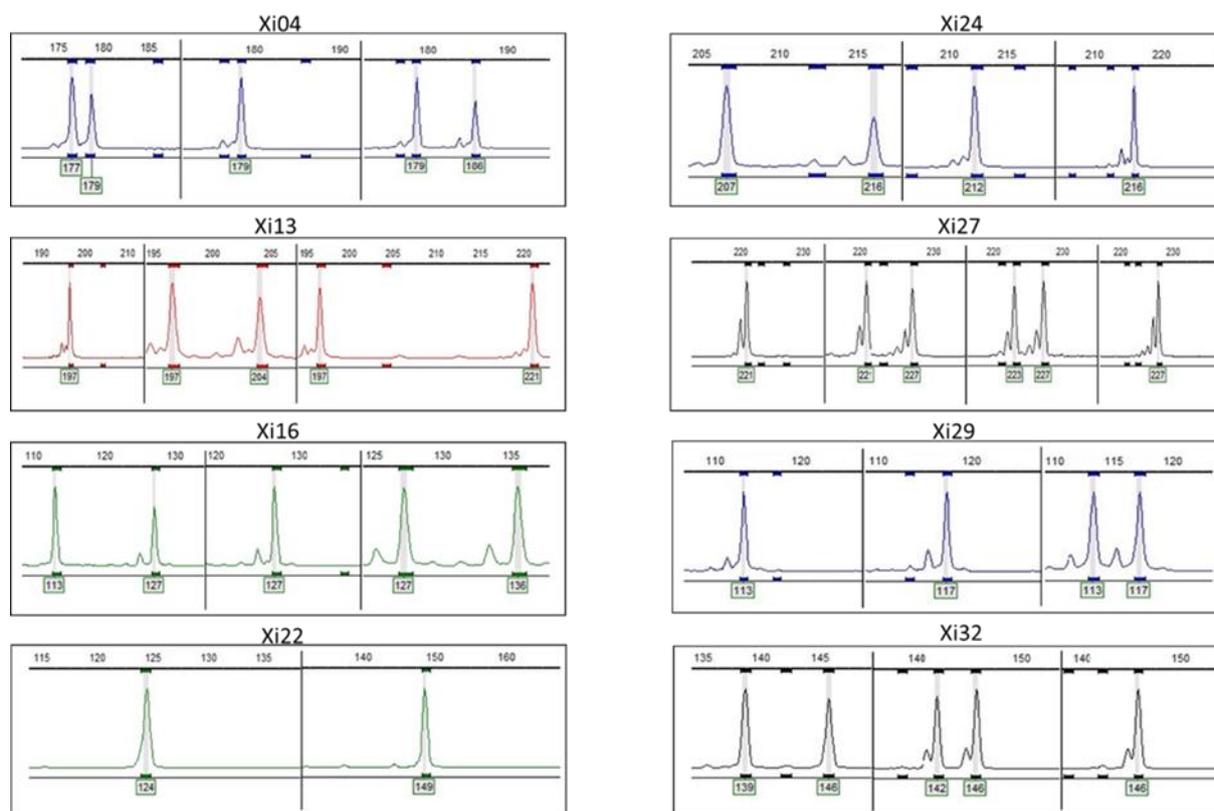


Figure 3.5. Electropherogram illustrating the signal of some alleles or combinations of alleles for the different microsatellite loci of *X. index*. Xi04, Xi13, Xi16, Xi22, Xi24, Xi27, Xi29 and Xi 32 are the names of the loci. Numbers under each peak are the sizes of alleles in base pairs.

Preparation of DNA templates from single individuals

DNA from a single nematode individual was isolated by a simplified procedure: a single adult or juvenile was hand-picked, placed in 0.5 ml PCR tube containing 50 μ l lysis buffer (KCl 50 mM, Gelatin 0.05 %, Tris pH 8.2 10 mM, Tween 20 0.45 %, Proteinase K 60 μ g/ml and MgCl₂ 2.5 mM). Then Eppendorf tubes were alternatively moved from liquid nitrogen to 60°C water bath for 10 times in order to facilitate break down of the nematode body. This step was followed by an incubation at 60°C for 90 min (the tubes were vortexed at least once during incubation to help breaking up tissues) and by a heating at 95°C for 10 min to inhibit the reaction of Proteinase K. Finally, individuals were cooled at 4°C, vortexed briefly (2-3 sec) and centrifuged shortly at 6,000 rpm for 30 sec. DNAs were stored at -20°C until use for PCR or further experiments.

Criteria of resistance evaluation and statistical analysis of the data

At each harvest date, aerial part of each plant was cut at the collar and each container was hermitically placed into a double plastic bag and transferred into a climatic chamber at 6 °C until soil nematode extraction. Resistance was evaluated primarily by the criterion ‘reproduction factor’ (RF) of the nematode, i.e. the ratio between numbers evaluated at a given plant age and the initial inoculum (200 total individuals = 50 individuals of each line x 4 lines). This ratio was calculated for each container (replicate) and mean of all replicates allowed to evaluate the RF for each accession. Accessions were classified as resistant (R) when their RF was lower than 1 and susceptible (S) when their RF was above 1. Weight of below-ground part of each plant replicate was also measured to evaluate ‘nematode numbers per gram of roots’. Data from each accession were statistically analysed by a one-way analysis of variance (ANOVA) using XLSTAT software, Paris, France (version 2014.5.03). Accessions were classified for resistance according to their actual RF values but statistical differences were evaluated after a $\log_{10}(x+1)$ transformation of nematode numbers (Noe, 1985). Means of transformed nematode numbers were then compared by the Fisher LSD multiple range test at $P \leq 0.05$.

III.1.3. Results

III.1.3.1. Evaluation of the resistance durability

The durability of nematode-resistant rootstocks and the cognate nematode adaptation has been studied in a 6-year assessment under controlled conditions.

Evaluation of resistance using the reproduction factor

Out of the six grapevine clones (8771, RPG1, RGM, 8624, CS and RdL), the S clones 8624 and RdL did not survive the attacks and died during the first two years. Therefore, we finally considered the 4 clones, 8771 (R), RPG1 (R), RGM (intermediate, I) and CS (S), for analysis over a 6-year period (3 to 6 years after planting, in cutting and *in vitro* material). Nematodes were recovered from soil containers and we evaluated their reproduction (RF= final nematode number/initial nematode number) per clone and type of multiplication. Development of the plants, as a complementary criterion, was estimated by the weight of roots.

The dynamics of nematode numbers differed according to the type of plant multiplication (Fig. 3.6). In cutting material, RFs of 8771 and RPG1 remained lower than 1 while they were intermediate in RGM and high in the susceptible control CS. Nevertheless, numbers tended to be higher in RPG1 (BC1 level) than in 8771 (F1 level), which agrees with the expected dilution of resistance factors in BC1 compared to F1 material. RFs of both R clones suggest that

resistance is stable. By contrast, in *in vitro* material, nematode numbers of both resistant clones were higher than 1 after 3 years and increased regularly and significantly beyond (Fig 4.6B). RFs of the intermediate clone RGM and the susceptible clone CS increased over years and were also much higher than in cutting material. Consequently, the type of material strongly influenced the resistance estimation at both F1 and BC1 levels. This dependence of resistance on the plant multiplication type is an original data that needs to be studied more deeply. The results obtained for *in vitro* material confirm the trend previously reported in Fig. 1.27 (Esmenjaud et al., 2010) for another F1 representative clone and for RPG1.

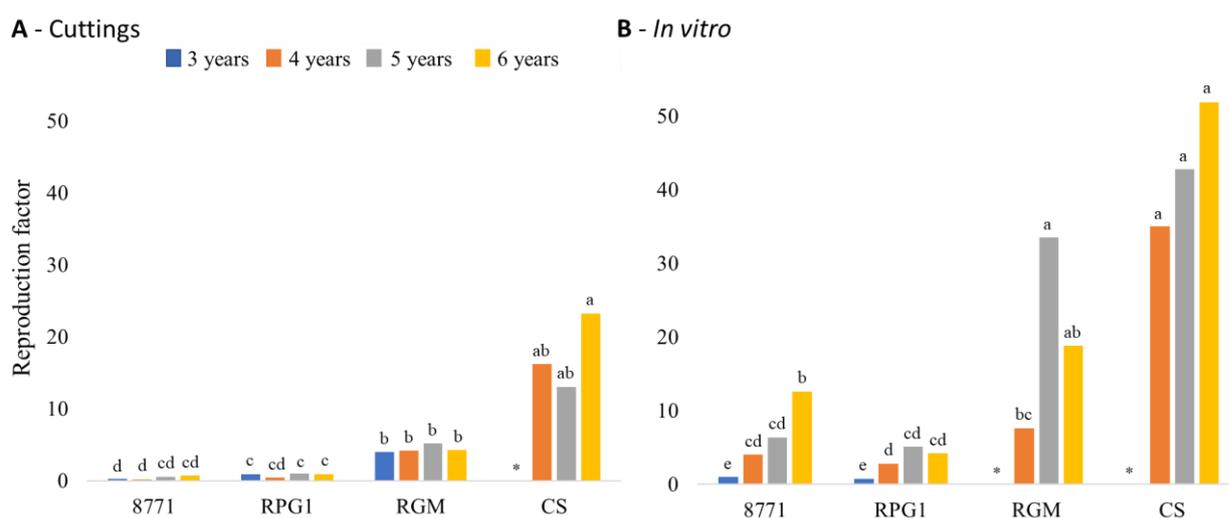


Figure 3.6. Reproduction factors (RF) to *X. index* for accessions 8771, RPG1, RGM, and CS after 3 (blue), 4 (orange), 5 (grey) and 6 (yellow) years. A: Cutting plants. B: *In vitro* plants. For each propagation type, bars with different letters significantly differ according to Fisher LSD test ($P \leq 0.05$). Black stars indicate missing data. In resistant material, there were at least 7 (8771) and 5 (RPG1) replicates. In RGM and CS, there were 3 to 5 replicates.

Evaluation of resistance using the nematode numbers per gram of roots

To evaluate the capacities of food supply for the development of *X. index* in different accessions (8771, RPG1, RGM and CS), we also recorded the total number of *X. index* per gram of roots (Fig. 3.7). It showed that, in cutting plants, numbers per gram of roots were much lower than in *in vitro* plants. After 6 years, the resistant accessions 8771 and RPG1 harbored 1.9 and 2.2 individuals per gram of roots, respectively. Meanwhile, RGM and CS harbored 6.9 and 39 individuals, respectively (Fig. 3.7A). In *in vitro* plants, CS had the highest numbers, i.e. 316 and 430 nematodes per gram of roots after 4 years and 6 years, respectively. The lowest numbers were 6.6 individuals (RPG1 after 6 years) and 20.2 individuals (8771 after 6 years) per gram of roots (Fig. 3.7B).

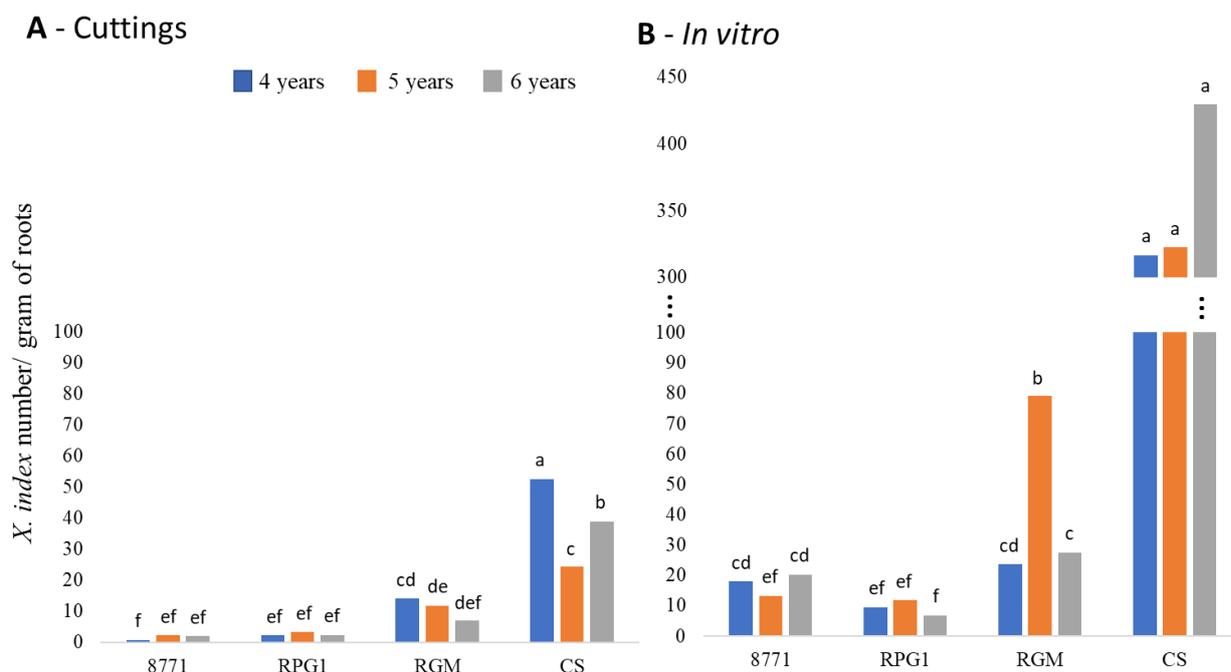


Figure 3.7. Nematode numbers of *X. index* per gram of roots on cutting plants (A) and *in vitro* plants (B). For each propagation type, bars with different letters significantly differ according to Fisher LSD test ($P < 0.05$). In resistant material, there were at least 7 (8771) and 5 (RPG1) replicates. In RGM and CS, there were 3 to 5 replicates.

We clearly observed that nematode densities in the roots of accessions RGM (intermediate) and CS (susceptible) were much higher than in the resistant accessions 8771 and RPG1. Nevertheless, as previously for RF values, densities in *in vitro* plants were always much higher than in cutting plants. Finally, results expressed from the numbers per gram of roots paralleled those obtained from the RF criterion. It must also be highlighted that, for the susceptible control CS, densities were 6 to 9 times higher in *in vitro* plants than in cutting plants.

Evolution of the nematode developmental stages

Our countings have evaluated the numbers of females, larva and male stages. We analyze hereafter their evolution over 3 years (4- to 6-year old plants).

Ratio 'female/larva'. In *in vitro* plants, the ratio was relatively stable whatever the age of the plants and ranged from 38.7 to 63.3 % (Fig. 3.8B). There was no major difference between resistant material, 8771 and RPG1, versus intermediate (RGM) or susceptible material (CS). Thus, the proportion was in average of 1.74 to 2.14 larva per female. By contrast, in cutting plants, we observed a different evolution. Four-year plants had values ranging from 41.0 to 76.8 % when all accessions are confounded while, in 5-year plants, the ratio increased in both resistant accessions and remained low in others. In 6-year plants, values were very high in 8771 and RPG1 (154.3 to 167.1%) and also increased in the other accessions to higher values (77.4

to 93.2 %) than in previous years (Fig. 3.8A). These values clearly showed an increased proportion of the female stage in plants aged of 5 (8771 and RPG1) or 6 years (all accessions). This increase resulted much more marked in the two resistant accessions. As the numbers of individuals recovered in resistant material has remained stable over the 3 years, these values state clearly that their respective populations have become older, which is in line with the resistant status of their accessions. Even though the phenomenon is less marked, the increased proportions of females in RGM and CS suggest that, in these plants, nematode numbers have reached a high and saturating density, which restricts the production of new progenies by the females. Interestingly, this clear aging of the nematode population in the material obtained from cuttings (undeniable at least in the resistant accessions) has not been observed in the material obtained from *in vitro*.

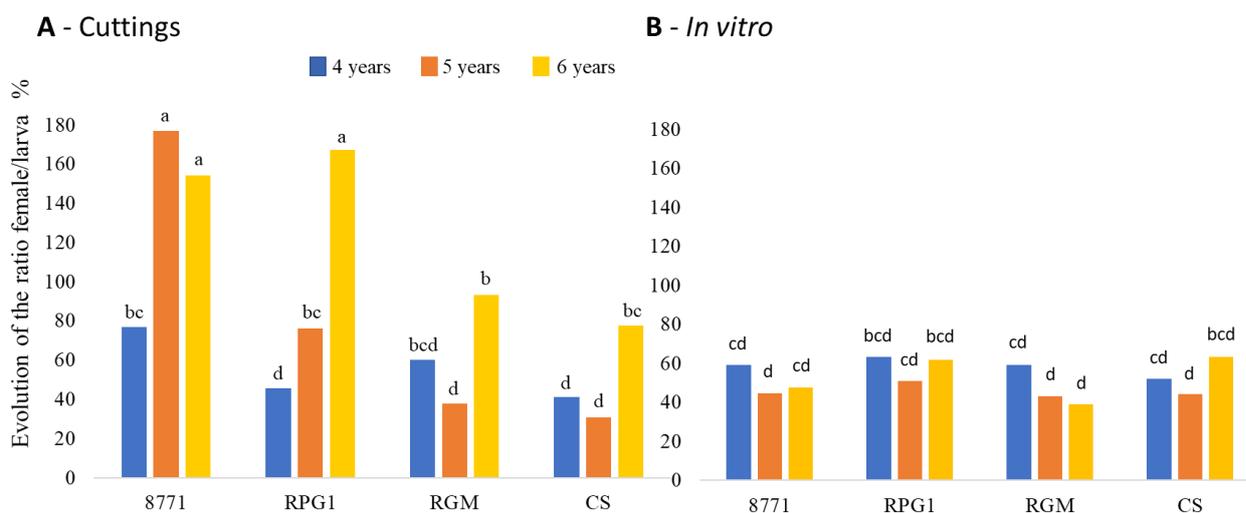


Figure 3.8. Evolution of the ratio female/larva of *X. index* on the 4 rootstock clones 8771, RPG1, RGM and CS obtained from cutting plants (A) and *in vitro* plants (B). Bars with different letters significantly differ according to Fisher LSD test ($P \leq 0.05$). Data from A and B have been mixed for statistical analysis.

The male numbers. We have recorded the male numbers over 4- to 6-year plants and picked out a total number of 13 males out of approx. 66,000 individuals. This represents a mean occurrence of 2 males per 10,000 nematodes (Table 3.4). We showed that there were very few males detected in the different accessions whatever the plant age and multiplication type. Consequently, it seems that males are found by chance (only 1 or 2 individuals per modality) and we did not evidence an effect of the accession (R or I or S) or the plant age (4 to 6 years) or the type of multiplication on the frequency of males (Table 3.4).

Table 3.4. Numbers of males of *X. index* recovered in different accessions at 4, 5 and 6 years

Year	Accession	Males number	Total <i>X. index</i> number	Frequency (per 10,000)
4 years	RPG1- cutting	2	1,088	18.4
	RGM - cutting	2	4,125	4.8
	RPG1- <i>in vitro</i>	1	4,804	2.1
	CS- <i>in vitro</i>	1	10,931	0.9
5 years	RPG1- cutting	1	2,354	4.2
	RGM - cutting	1	5,160	1.9
6 years	RPG1- cutting	1	2,311	4.3
	8871- cutting	1	1,331	7.5
	RGM- cutting	1	4,249	2.4
	8771- <i>in vitro</i>	1	28,337	0.4
	8624- <i>in vitro</i>	1	1,317	7.6
Total		13	66,007	1.97

III.1.3.2. Evaluation of the nematode adaptation

As explained before, the principle of this experiment is to monitor simultaneously the putative adaptation of the nematode to the resistance and to get data about the hypothetic genetic mechanism of this phenomenon.

Evaluation of the relative abundance of nematode lines

For each plant multiplication type and on each year (from 3 to 6 years), we determined the line identity of representative nematode individuals. In each replicate (pot), we sampled randomly 5 individuals in 2015 and 2016 and 8 individuals in 2017 and 2018. We analyzed their

multilocus genotypes (see Table 3.3 for total numbers of plants per propagation type, accession and age). All additional nematodes extracted were kept at -20°C.

Confirmation of the identification of the genotypes from sampled individuals. We could successfully attribute the high majority of individuals to a given inoculated line. In rare cases, we did not obtain the signal in all loci of an individual as the alleles of 1 to 2 loci were poorly expressed (low peak signal) or completely absent (Fig. 3.12). In such cases, the other loci with unambiguous signals allowed to attribute them to a given line. Exceptionally, alleles from 3 loci were lacking and we considered that these individuals are missing data. Individuals that could not be attributed to a line were analyzed with more attention to establish whether they were hybrid individuals.

Evolution of the line frequency in the different accessions over the 3- to 6-year experiment.

We recovered individuals from all four lines (Italy, Spain, Samos and Iran) in the nematodes sampled on plants obtained from both cutting and *in vitro* plants (Fig. 3.9) over the 3- to 6-year experiment. Absence of certain lines in replicates was exceptional and presumably due to chance because of low numbers of individuals genotyped and not to a real lack. For example, in the few *in vitro* RGM plants genotyped, we recorded 2 lines (Italy and Spain) out of 3 individuals after 3 years, 3 lines (Italy, Spain and Samos) out of 15 individuals after 4 years, and the 4 lines out of 23 individuals and 3 lines (Italy, Spain and Samos) out of 20 individuals after 5 and 6 years, respectively (Fig. 3.9B). Nevertheless, in an overall view, Iran and Italy were the lines most frequently recovered and appeared to express an higher aggressiveness than the two others (Fig. 3.9 A&B).

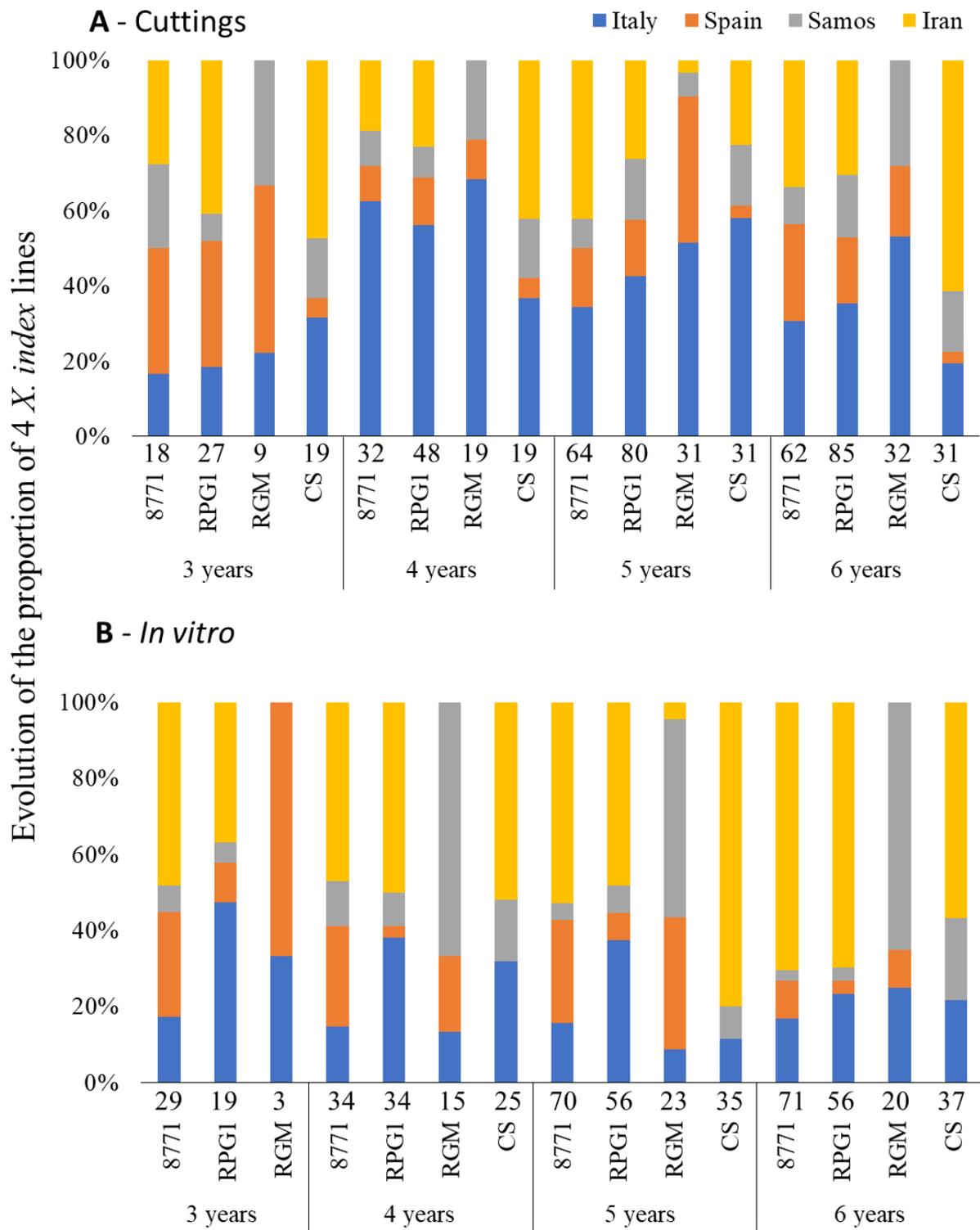


Figure 3.9. Evolution of the proportion of the four nematode lines (Italy, Samos, Spain and Iran) of *X. index* during the durability experiment over 3 to 6 years on the grapevine clones, 8771, RPG1, RGM and CS. A: Cutting plants. B: *In vitro* plants. The numbers below each bar indicate the total nematode numbers that were genotyped.

We then focused more precisely on the data obtained with the resistant accessions 8771 and RPG1 after 6 years to highlight the evolution of the line frequencies between the inoculation (25 %) and the end of the experiment. Chi² (χ^2) tests showed that we cannot retain the hypothesis that frequencies of each line are equivalent (Table 3.5). The predominance of lines Iran and Italy is confirmed as illustrated in Fig. 3.10. The line Iran resulted particularly predominant in the plant material obtained from *in vitro*.

Table 3.5. Distribution of the numbers of individuals of *X. index* assigned to each line, after 6 years, in the resistant accessions 8771 (F1) and RPG1 (BC1).

Lines	8771		RPG1	
	MIV	Cuttings	MIV	Cuttings
Italy	12	19	13	30
Spain	7	16	2	15
Samos	2	6	2	14
Iran	50	21	39	26
Total number	71	62	56	85
Expected number*	17.75	15.50	14.00	21.25
Threshold value (P< 0.01)	11.35	11.35	11.35	11.35
χ^2 (3 DoF)	80.94	8.58	65.30	8.98
Probability H0**	P< 0.01	P< 0.05	P< 0.01	P< 0.01

* The number expected for each line is 25 % of the total number in the hypothesis that all lines have an equivalent frequency (aggressiveness)

** H0 hypothesis = no difference in frequency

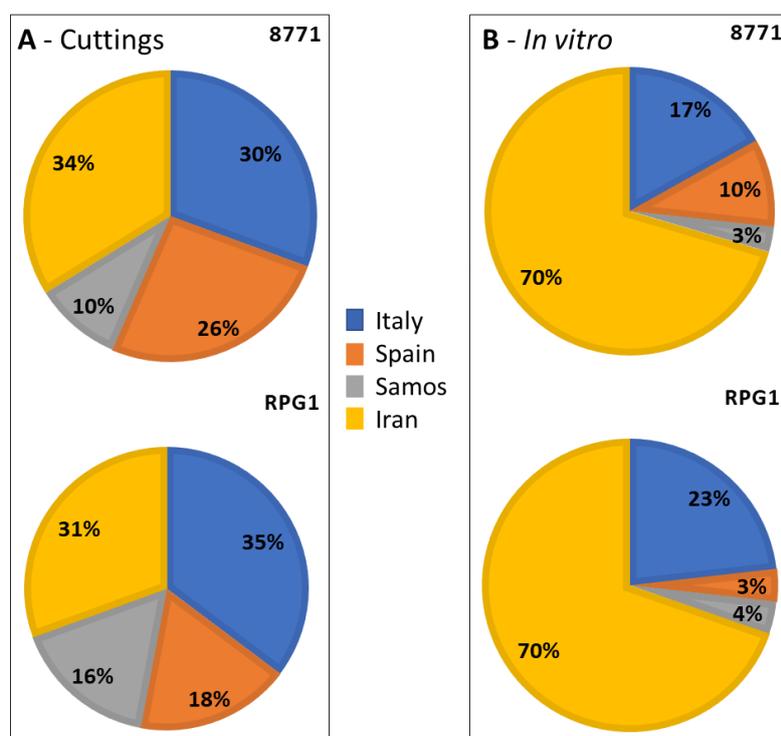


Figure 3.10. Relative frequency of each line (Italy, Spain, Samos and Iran) of *X. index* in the two resistant accessions 8771 and RPG1 after 6 years. A: Cutting plants. B: *In vitro* plants.

Detection of hybrid individuals

Detection in 3- and 4-year old plants. Search of hybrid individuals has begun in plants aged 3 years, i.e. one year earlier than nematode countings for resistance evaluation. A total of 485 individuals (200 individuals in 3-year plants and 285 individuals in 4-year plants) have been genotyped for microsatellites over the two first years for both cutting and *in vitro* plants (5 individuals for each replicate = pot). From these data, the first (and sole) evidence of a hybridization event was found at 4 years post-inoculation. This hybrid individual has been detected from an *in vitro* RPG1 plant and is a cross between lines 'Iran' and 'Italy'. This detection of the hybrid individual between Iran and Italy is illustrated in Fig. 3.12. The figure shows the presence of new allelic associations such as alleles of 124 bp and 149 bp in locus Xi22 and alleles of 212 bp and 216 bp in locus Xi24. This figure also demonstrates how we can distinguish reliably this hybridization phenomenon from accidentally mixed DNAs in these lines.

Detection in 5- and 6-year old plants. In 5-year old plants, we monitored 488 individuals and obtained 9 hybrid individuals. In 6-year old plants, 11 hybrid individuals were detected among 496 *X. index*. These 20 hybrids ranged into crosses involving several lines: Iran versus Italy, Samos vs Spain and Italy vs Spain (Fig. 3.11). Interestingly, in 6-year old plants, most hybrids that we detected have the line Spain as one of their parents. In 5- and 6-year old plants, we

found some hybrids with low or no allelic signals at locus Xi27. Nevertheless, based on the rest of the alleles and the evidence for several particular allelic combinations, we could state undeniably the occurrence of interspecific hybridization events. Nevertheless, for one of the hybrids (marked 'Iran vs ???'), we could identify Iran as one of the parents, but we failed to identify the other parent presumably because of the absence of the expression of its specific alleles at certain loci (Fig. 3.11).

		Locus names		Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8	Number of individual																
		Allele		Xi29 (GA)	Xi04 (TG)	Xi16 (TC)	Xi13 (CA)	Xi24 (AC)	Xi22 (AAAC)	Xi32 (GTT)	Xi27 (GT)																	
Years post inoculation	Plant types	Original descendency lines	Line	113	117	177	179	186	113	127	136	197	204	221	207	212	216	124	149	139	142	146	221	223	227			
			Spain	113	117	177	179		127	136	197							216	124			142	146			227		
			Italy	113			179	186		127	136	197	204					216	124				146	221			227	
			Samos		117	177	179		127		197	204		207				216	124				146		223	227		
			Iran	113			179		113	127		197		221		212				149	139		146	221				
4	<i>In vitro</i>	RPG1	Iran vs Italy	113			179		113	127		197	204			212	216	124	149	139		146	221		227	1		
5	Cuttings	RGM	Samos vs Spain		117	177	179			127	136	197					216	124			142	146			227	1		
		RdL	Italy vs Spain	113			179	186		127	136	197	204				216	124				142	146	?	?	?	1	
		8771	Iran vs Italy	113			179			113	127	136	197		221		212	216	124	149	139		146	221			1	
	<i>In vitro</i>	CS	Hybrid between	Iran vs Italy	113			179		113	127		197		221		212	216	124	149	139		146	221			3	
			Samos vs Spain	113	117	177	179		127		197	204						216	124				146		223	227	1	
			Samos vs Iran	113	117	177	179		127		197	204		207				216	124				146	221		227	1	
			8624	Samos vs Spain	113	117	177	179		127		197	204	207				216	124				142	146		223	227	1
	6	Cuttings	RPG1	Italy vs Spain	113	117		179	186		127	136	197	204				216	124			142	146	221		227	1	
			CS	Iran vs ???	113			179		113	127		136	197			212	216		149	139		146	221		227	1	
			RdL	Italy vs Spain	113			179	186		127	136	197	204				216	124				142	146	221		227	2
<i>In vitro</i>		RGM	Hybrid between	Italy vs Spain	113			179	186		127	136	197	204			216	124				142	146	221		227	1	
			Italy vs Spain	113			179	186		127	136	197	204				216	124				142	146	221		227	1	
			8624	Italy vs Spain	113			179	186		127	136	197	204				216	124				146	221		227	1	
			Samos vs Spain	113	117	177	179		127		197	204		207				216	124				142	146		223	227	1
			Samos vs Spain		117	177	179		127		197	204		207				216	124				142	146	?	?	?	1
			Iran vs Spain	113			179		113	127		197		221		212	216	124	149				142	146	?	?	?	1
			CS	Samos vs Spain		117		179		127		197		207				216	124				142	146		223	227	1
Iran vs Spain	113			179		113	127		197		221		212	216	124	149				142	146	?	?	?	1			

Figure 3.11. Evidence of microsatellite multilocus hybrid genotypes detected from the four *X. index* lines mixed in containers of plant material obtained from cutting and *in vitro*. '?' indicates the absence of an amplification signal.

Synthesis on 3- to 6-year old plants. In our results, we could detect a total number of 21 hybrid individuals. At least one event has been detected in each grapevine variety (8771, RPG1, RGM, 8624, RdL and CS). Hence, this hybridization event does not appear specific to certain clones and is not linked with the resistance status of the material. Even though we have only few hybridization cases, the hypothesis that the resistant plants (e.g. 8771 and RPG1) might induce a selection pressure that favors the production of crossbreds between the lines is *a priori* not supported by our results. The frequency of the detection of hybrids varied deeply over years: from none out of 200 individuals after 3 years, it hardly increased to 1 out of 285 individuals after 4 years (3.5 per 10,000) and then moved to 9 out of 488 individuals after 5 years (184 per 10,000) and remained relatively stable with 11 out of 496 individuals after 6 years (221 per 10,000). The contribution of each line to the hybridization event was relatively equivalent after 5 years, ranging from 0.2 (Samos and Spain) to 0.3 (Italy and Iran), but Spain became dominant at 6 years (0.45) preceding all other lines with a contribution from 0.14 (Iran and Samos) to 0.23 (Italy).

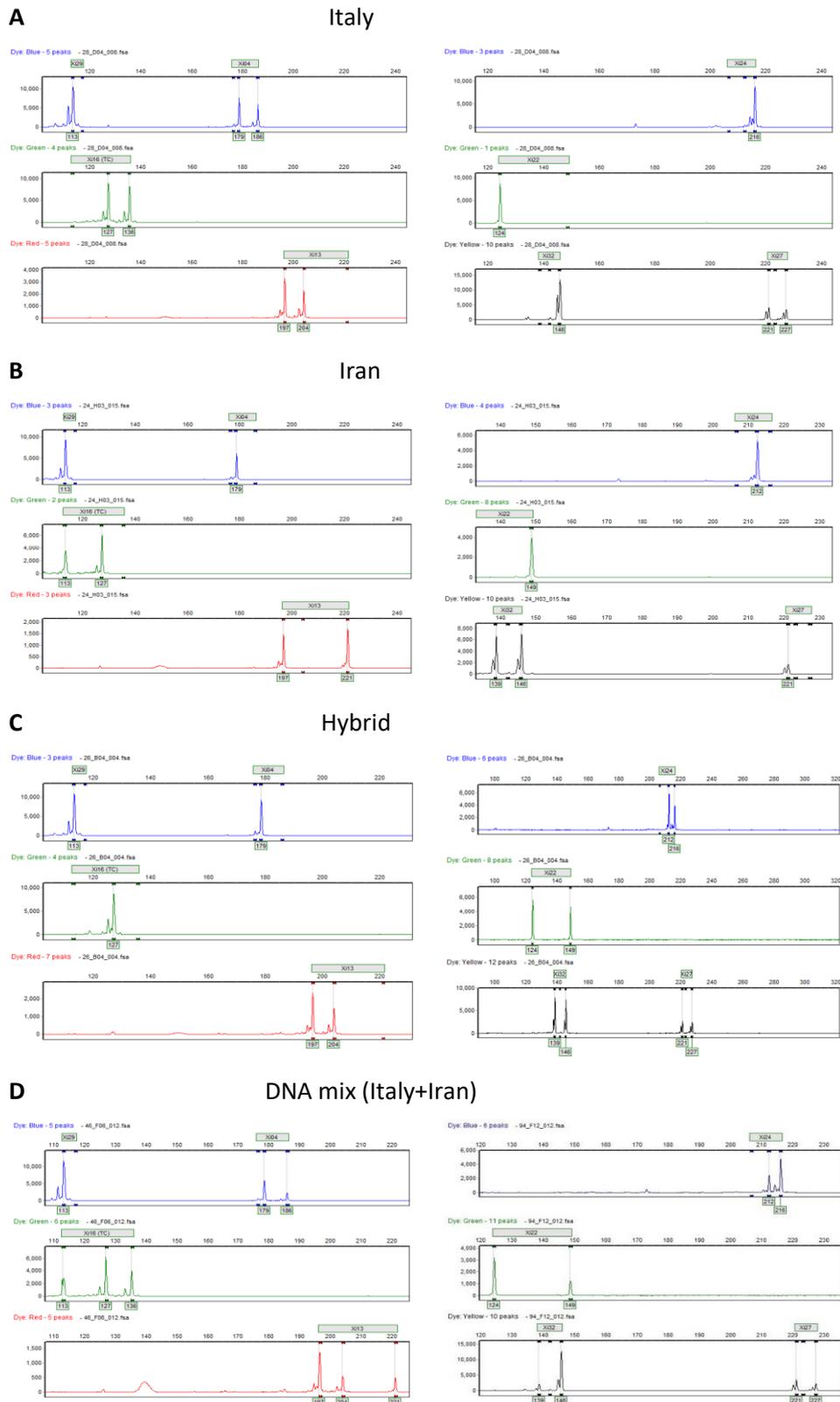


Figure 3.12. Electropherogram illustrating the detection of a hybridization event (C) between single individuals from the lines Italy (A) and Iran (B), respectively. D shows the control DNA mix between single individuals from Italy and Iran. Graphs of all 8 microsatellite loci (named above each locus in green rectangles) considered in the study are represented.

III.1.4. Discussion and conclusion

III.1.4.1. Evaluation of the resistance durability

Materials obtained from *in vitro* and cuttings show contrasting host suitabilities to *X. index*.

In our experiment with an initial *in vitro* multiplication type, we have observed an increase of the *X. index* RFs on the resistant material 8771 (F1) and RPG1 (BC1). Indeed, in 8771 and RPG1, RFs were, respectively, 6.38 and 5.12 after 5 years and 12.59 and 4.22 after 6 years. For comparison, after 4 years the results previously obtained by Esmenjaud et al. (2010) in another F1 accession, the *Muscadinia* (R) x *Vitis* (S) cross VRH 97-99-79, had shown a RF of 5.27 and, thus, of equivalent value, which is in line with our current data with 8771. Our data are also in overall agreement after 4 years with those obtained for RPG1 which had a RF slightly higher of 7.57. These RFs resulted significantly higher than 1 and indicated an at least partial breakdown of resistance. Susceptible (CS) and intermediate materials (RGM) have shown high RFs and might have reached a maximum after 6 years. Results from these accessions are in line with the previous results (Esmenjaud et al. 2010) with control accessions RGM and RL. By contrast, in the experiment performed from cutting plants, RFs varied in a reduced interval between 0.14 and 0.67 in 8771 and between 0.44 and 0.94 in RPG1 for the period from 3 to 6 years (Fig. 3.6). RFs always remained lower than 1. Thus, in resistant accessions, this phenomenon of putative adaptation of the nematode to the resistance for plants obtained from *in vitro* has not been observed for cuttings. No previous comparative evaluations have been performed between *in vitro* and cutting materials and our results are original. Susceptible and intermediate control materials have shown, as expected, high RFs over years.

***In vitro* origin of plant material modifies the resistance to *X. index*.** Our results clearly show that the *in vitro* initial conditions of the plant material have influenced the plant host suitability to *X. index* by facilitating the nematode multiplication. It is hypothesized that the previous continuous *in vitro* culture of the plant material modifies the resistance to *X. index*. This may be linked to the modification of the root architecture in the plants obtained from *in vitro* in comparison with plants rooted from cuttings. Roots systems from *in vitro* have a more compact shape and produce a higher density of rootlets. The use of the rooting exogenous hormone IBA before the experimental set-up in soil might also be involved in the modification of resistance. Nevertheless, the effect of IBA in the *in vitro* rooting medium, studied in the interaction between *Prunus* plants and root-knot nematodes and added at the same rate as in our durability experiment (0.5 ppm), did not alter the behavior of *P. cerasifera* accessions, carrying the *Ma* gene for resistance to *M. arenaria*, 70 days after plant transfer into a sandy soil (Esmenjaud et al., 1993; 1996).

Resistance and structure of the population appear related. The ratio 'female/larva' seems to be linked to the resistance phenomenon. We can put forward that, in plants showing a low RF, the female proportion increases, which corresponds to an aging of the population. This can be explained by the long-life duration of the female stage. Indeed, the dagger nematode *X. index* has been shown to survive for more than 4 years, while starving in the soil without plant host (Demangeat et al., 2005). In field soils, the actual survival of adult nematodes is estimated to be of 7 years from the work conducted in the field by Vuittenez et al. (1969). An at least 5-6-year survival may explain why nematodes are still recovered in our experiments in pots containing 6-year old resistant plants obtained from cuttings. In resistant plants obtained from *in vitro*, adult nematodes are presumably a mix i) of female individuals that were already at this developmental stage at the time of inoculation and ii) of younger female individuals originating from larval stages that have been able to undergo their development and complete their life cycle. Knowing the proportion of ancient-female and neo-female adults would allow a better interpretation of our results. In our experimental conditions, it would also be worth knowing the real survival potential of *X. index* individuals kept starving (without host plant) in the soil container.

Male occurrence does not appear to be linked with resistance status. We have observed a rare occurrence of males (mean value of approx. 2 per 10.000), whatever the resistance status of the plant material. Even though counting males is difficult and subject to misvaluation when dispersed among high female numbers, their evaluation is more reliable when few nematodes develop such as in resistant plant material obtained from cuttings. Nevertheless, even in this case, rare males were recovered. It thus appears that male occurrence is not linked with the population density. The increase of the male percentage shown in the case of RKNs facing a poor host or having reached very high densities is not observed for the dagger nematode *X. index*. Nevertheless, this suggests that a low basic proportion of males exists in the population whatever the plant status. The proportion that we have obtained confirms the very rare occurrence of males previously reported by many authors (Dalmasso, 1970; Luc & Cohn, 1982; Siddiqi, 1986). Nevertheless, an exception has been reported in Australia with a proportion of males as high as 1-2 % (Harris, 1977).

III.1.4.2. Occurrence of hybridization events in the populations

The first occurrence of hybrid individuals in *X. index*, previously known as being a facultative-meiotic parthenogenetic species (Dalmaso & Younes, 1969) has been reported recently in the field (Villate et al., 2010). In this work conducted in Bordeaux vineyards, some hybrids could be detected in samples located in the area overlapping two genotypically different groups of individuals from presumably distinct introductions in a same field. Our results have shown for the first time the occurrence of such hybridization events under controlled conditions. We have successfully designed an experiment that allowed to evidence this phenomenon. The characterization and rearing of appropriate single-female descendencies has permitted to create lines from which both the line identity and the hybrid occurrence could be traced over several years. Moreover, our numbers of genotyped individuals were high enough for the detection of hybrids.

We analyze hereafter the putative factors that may either have been associated to, or have led to, or have influenced this occurrence.

Relationship between hybrids and the resistance status of the plant. We would have expected that males appear in high numbers in resistant material to facilitate recombination events and favor the occurrence of individuals carrying virulence factors in order to overcome the resistance. Our results do not support this hypothesis and suggest that hybrids are recovered independently of the plant resistance status. It is probable that hybrids might appear in all conditions and that their numbers might be proportional to the population density. This would explain why we have detected a higher number of hybrids in susceptible or intermediate accessions such as CS, RL and RGM than in resistant accessions such as 8771 and RPG1.

Relationship between hybrids and the age of plants. We could show that the occurrence of hybrid events increased from a single individual after 4 years to 9 and 11 individuals after 5 and 6 years, respectively.

Relationship between hybrids and the type of plants. No clear difference has been observed between plants obtained from cuttings or *in vitro*. The difference between young and old plants appears independent from the multiplication status of the plant.

Relationship between hybrid and male numbers. We did not evidence any link between hybrid and male numbers. Nevertheless, as it did not aim at studying the male frequency, our experimental design was not adapted to establish the putative relationship between these two features.

III.1.4.3. Consequences of the partial breakdown of resistance to *X. index* in plant material obtained from *in vitro*

We can take into account two complementary aspects. We can consider first the plant side and foresee the consequences in the use of resistant plants obtained from *in vitro*. Then we can consider the nematode side and discuss about the putative adaptation of the nematode facing resistance factors influenced by this *in vitro* historical background.

Evolution of the interaction viewed from the plant side. The results that we have obtained strongly suggest taking care when using *in vitro* material in greenhouse or field experiments. It seems that the plant initially grown *in vitro* has become more susceptible to the nematode for several years. The plant keeps a memory of its initial history and its interaction with the nematode is modified for a long duration. This exhorts the researchers to avoid the use of such plant material for studies on 'natural' or 'field' plant-nematode interactions. Growers should avoid using such material in order to reduce their nematode reservoir in the soil.

Evolution of the interaction viewed from the nematode side. The ratio female/larva fits with the hypothesis that a 'normal' or 'natural' resistance does occur in cutting plants. In 8771 and RPG1 the populations seem to become 'older' by comparison with *in vitro* plants. This ratio after 6-year inoculation also increased slightly in RGM and CS, which may be consecutive to high nematode densities in the limited space or/and food resource available from containers with aged plants. These results should discard the hypothesis of a phenomenon of nematode genetic adaptation. It appears probable that the plant physiology is durably modified (i.e., over a duration of at least 6 years) in material previously grown *in vitro*. The better host suitability of the plant may render it more susceptible to the nematode. In this hypothesis, no particular nematode genetic adaptation is expected and, consequently, those nematode individuals raised on material obtained from *in vitro* would not have acquired new virulence factors. Nevertheless, demonstrating this would need to compare the reproduction of these putatively resistance-adapted individuals with individuals that have never been faced to resistant plant material.

Hybridization does not appear as a key factor for overcoming resistance. Hybridization can contribute to adaptation and speciation in populations (Burke & Arnold, 2001). In our experiment, as hybrid individuals were recorded apparently whatever the nematode line and the plant accession, resistance may not favor the occurrence of hybrid events. Hence, there was no evidence that hybridization is a key feature for the adaptation of *X. index* to resistance over time. In moderate and susceptible plants, we may hypothesize that hybridization is consecutive to the selection pressure exerted by the rarefaction of food supply from root tips.

Lines apparently express differences in aggressiveness. Previous work conducted at ISA (Demangeat et al. 2010) reported variable reproduction rates of *X. index* among lines that had

been obtained from Cyprus, California and Spain and illustrated differences in their aggressiveness. Our experiments are in overall agreement with these results by showing that the two lines Iran and Italy express a higher aggressiveness than Spain and Samos. Nevertheless, despite the 6-year duration of the experiment, none of the four mixed lines appeared to exclude totally the others.

III.2. RESISTANCE DURABILITY BASED ON A LONG-TERM ASSESSMENT UNDER FIELD CONDITIONS

III.2.1. Introduction

Resistance durability studies are logically long to perform and, in order to provide results, they must be planned early. In the field, a single trial dates back before 2000. This experiment has been installed from 1999 in the experimental unit of Domaine du Chapitre in a grapevine area known as highly infected by *X. index* and inducing very diverse and marked symptoms of GFLV. In this experiment, GFLV evolution has been monitored by INRA Montpellier, Montpellier SupAgro and ITV.

The experimental design aimed to evaluate the behavior of different rootstocks against GFLV transmitted by *X. index*. These were the BC1 rootstock RPG1 (Nemadex AB) in comparison with three other rootstocks, SO4, Ruggeri 140 (140Ru) and Borner. Results that we have obtained and reported within this thesis deal with the nematode numbers from a soil sampling performed in June 2015.

III.2.2. Plant material and experimental design

III.2.2.1. Plant material

RPG1 and the three rootstocks, SO4, 140 Ruggeri (140Ru) and Borner have been considered. The SO4 rootstock (an hybrid between *V. berlandieri* and *V. riparia*) is very sensitive to GFLV and *X. index* (Goldammer, 2018). It shows a good ability to grafting and to cutting production. The 140 Ruggeri rootstock (*V. rupestris* x *V. berlandieri*) is characterized by a good development in calcareous soils and thus by a good adaptation to lime chlorosis (Goldammer, 2018). The Borner rootstock (*V. vinifera* x *V. cinerea*) has been selected in Germany as unfavorable to the multiplication of GFLV (Ruhl, 1996). RPG1 (Nemadex AB) has been obtained from the cross between the F1 *Vitis-Muscadinia* hybrid VRH 8773 and 140Ru (see Fig. 1.26 for more details). Nemadex AB has been grafted with either Caladoc or Cabernet-Sauvignon; 140Ru has been grafted with Caladoc while SO4 and Borner have been grafted with Cabernet-Sauvignon (Fig. 3.13).



Figure 3.13. Design of the field experiment of Nemadex AB in Domaine du Chapitre (Villeneuve-les-Maguelonne) (Source: INRA-IFV). Four different rootstocks were used Nemadex AB (Nem), 140 Ruggeri (140Ru), SO4 (SO4) and Borner (Borner) grafted on either variety Caladoc (Cal; rows 1 and 2) or variety Cabernet-Sauvignon (CS; rows 3 to 5). Rootstock-variety assemblies were: Nem:Cal, 140Ru:Cal, SO4:CS, Nem:CS and Borner:CS. Each replicate is composed of 6 plants indicated by the same color and is designated by its number placed after the rootstock-variety assembly.

III.2.2.2. Experimental design

The experimental design (Fig. 3.13) contains five parallel grapevine rows planted in 1999 and divided in two parts as follows. i) The first part (Caladoc part) was planted on the two adjacent rows located in the border of the grapevine field in an area which was less contaminated by *X. index*. This area has been planted with the variety Caladoc grafted on either Nemadex AB or 140Ru rootstocks. There were 12 replicates of 6 plants for each rootstock-variety assembly. ii) The second part (Cabernet-Sauvignon part) was planted on three adjacent rows with the variety Cabernet-Sauvignon (CS) grafted onto either Nemadex AB, or SO4, or Borner rootstocks. There were 16 replicates of Nemadex AB, 18 replicates of SO4 and 5 replicates of Borner. As previously, each replicate contained a set of 6 consecutive plants.

III.2.3. State of the art in June 2015

III.2.3.1. Plant development

Plant development data have been recorded by IFV (Domaine de l'Espiguette, Le Graud-du-Roy). GFLV expressed a very marked effect on the plant development. Fig. 3.15 illustrates the difference between plants from a replicate of CS grafted onto the GFLV susceptible rootstock SO4 in comparison with plants from the two adjacent replicates where CS is grafted onto Nemadex AB. Whereas CS grafted on Nemadex AB was vigorous and apparently healthy, CS grafted on SO4 showed typical viral GFLV symptoms such as stunting, fanleaf, short internodes, limited shoot growth... A similar viral decline phenomenon was also recorded in 140Ru rootstock and in a more limited extent in Borner rootstock. In the Caladoc part, GFLV symptoms were less marked than in the CS part. Plant development was also much better in the Caladoc part than in the CS part; e.g. in the first case the percentage of dead plants was 4.2% while it was 16.7% in the second part (Fig. 3.17). The highest percentage of dead plants were recorded in SO4 rootstock (28/108; 25.9%), followed by Borner (10/30; 33.3%), 140Ru (5/72; 6.9%) and Nemadex AB (2/168; 1.2%).

III.2.3.2. ELISA GFLV infection

The distribution of GFLV infection has been monitored by ELISA tests conducted by IFV and the situation on the date of soil samplings is reported in Fig. 3.17. The percentages of ELISA positive plants from 2000 to 2014 in the CS part (Fig. 3.14A) illustrate the dramatic retardation of the contamination of Nemadex AB in comparison with SO4 (Figs 3.14A & 3.15). This retardation is less marked in the Caladoc part (Fig. 3.14B).

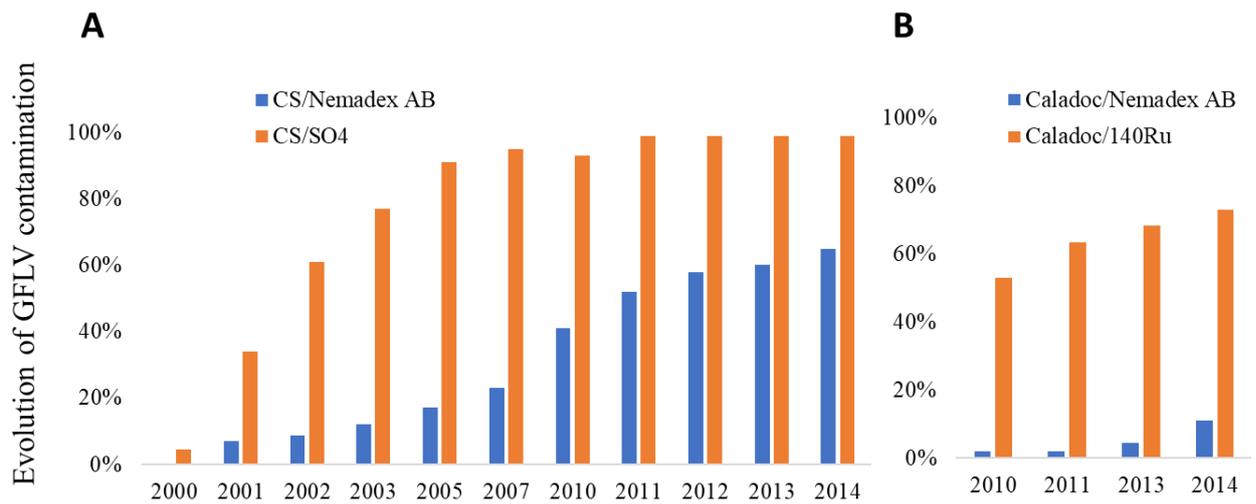


Figure 3.14. Evolution of GFLV contaminations by ELISA tests in the experiment of Domaine du Chapitre planted in 1999. A: Cabernet-Sauvignon part: CS/Nemadex AB and CS/SO4 from 2000 to 2014. B: Caladoc part: Caladoc/Nemadex AB and Caladoc/140 Ru from 2010 to 2014 (Source: INRA-IFV).

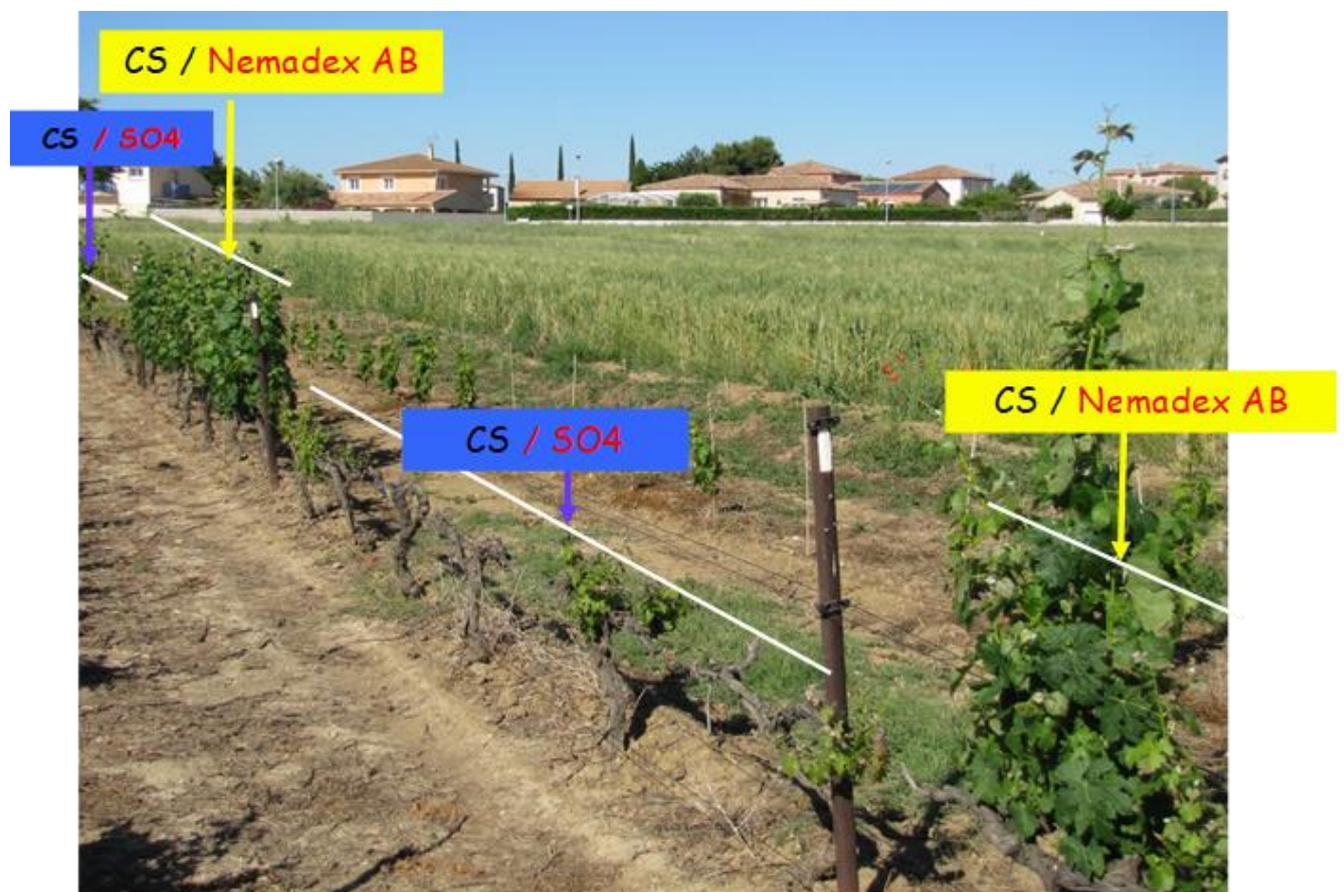


Figure 3.15. Differential GFLV attacks in Nemadex AB and SO4 rootstocks grafted with Cabernet-Sauvignon in the field trial, 12 years after planting (Photo credit INRA Sophia Antipolis, 2011).

III.2.4. Evaluation of nematode numbers collected in 2015 (after 16 years)

III.2.4.1. Materials and methods

Procedure for soil sampling

Soil samples were taken in each of the replicates (i.e. 63 total samples) in trenches, approx. 60 cm deep, 120 cm long and 40 cm wide, dug by a narrow excavator (Fig. 3.16). Sampling excavation by the mechanical digger was performed in the middle part of each replicate, i.e. between plants 3 and 4 out of the 6 consecutive plants, on the South-exposed side of the row and was thus considered as representative of the replicate. Samples were made from soil clods recovered 50-60 cm deep on the side of the trench located the closest to the plant row. Two sub-samples, approx. 2 kg each, were recovered from each trench (Fig. 3.16). Immediately after sampling, the volume of soil collected in each trench was handled gently and placed in an ice box to protect it from heat and desiccation until its storage at 4-5°C at UMR ISA.



Figure 3.16. Soil sampling in the field in trenches, approx. 60-cm deep, 120-cm long and 40-cm wide, dug by a narrow excavator in June 2015. Sampling was performed in the middle part of each replicate (between plants 3 and 4 out of the 6 plants). In each trench, 2 sub-samples of 2 kg of soil were taken by hand.

Procedure for extraction

Nematode extraction aimed at detecting *X. index* in priority. It also aimed at detecting *X. pachtaicum*, a *Xiphinema* species commonly found in vineyards. Extractions were performed using the method described previously for *X. index* under controlled conditions.

Statistical analysis

Statistical analysis has been performed as previously mentioned in the first part (controlled conditions) of this chapter i.e. in the paragraph 'Criteria of resistance evaluation and statistical analysis of the data' (IV.1.2.2 Methods).

III.2.4.2. Results

Detection of *X. index*

Nematode numbers. In 2015, i.e. 16 years after grapevine planting, extractions from soil samples collected 50-60 cm deep allowed to evaluate nematode numbers. Very low numbers were recovered, i.e. 21 *X. index* individuals in total (14 individuals from the first 2-kg sub-sample and 7 individuals from the second 2-kg sub-sample) when all the rootstock:variety assembly are confounded. Eleven out of the 63 samples contained *X. index* and numbers ranged from 1 to 6 individuals per 4 kg of soil. No nematodes were recovered in the Caladoc part of the assay (comparison between Nemadex AB and 140Ru). In the CS part, comparison of three rootstocks showed that the highest numbers were recovered in SO4, followed by Borner (on only 5 replicates) and Nemadex AB. A single nematode has been recovered from the 28 total Nemadex AB samples (112 kg of soil) in the experiment.

An ANOVA analysis, conducted on CS experiment showed, after a $\log_{10}(x+1)$ transformation of data, a significant difference between SO4 and Nemadex AB but not between Borner and both other rootstocks (Table 3.6). Another ANOVA analysis conducted on all rootstock:variety assemblies also confirmed these results after transformation by stating that Nemadex AB:CS had significantly lower numbers than other assemblies except Borner:CS that resulted intermediate and non-significantly different from others (Table 3.7).



Figure 3.17. Spatial distribution of ELISA GFLV-positive, negative and dead plants in 15-year-old grapevine plants (2014). GFLV-positive plants are in pink, the GFLV-negative plants in green, and the dead plants in grey (Source: INRA-IFV). The small brown boxes indicate the location of the sampling holes dug along the rows between two plants (plants 3 and 4).

Table 3.6. ANOVA analysis of *X. index* numbers (individuals per kg of soil) in the rootstocks Börner, Nemadex AB and SO4 grafted with Cabernet-Sauvignon. (A) ANOVA parameters for $\log_{10}(x+1)$ transformed nematodes numbers. (B) Mean comparison with the LSD Fischer test on transformed nematode numbers at $p>0.05$.

(A)

Source	DoF	Sum of squares	Mean of squares	F	Pr > F
Model	2	0,064	0,032	3,886	0,030
Error	36	0,299	0,008		
Total	38	0,363			

(B)

Modality	Mean	Groups	
SO4: Cs	0,093	A	
Borner: Cs	0,045	A	B
Ném: Cs	0,006		B

Table 3.7. ANOVA analysis of *X. index* numbers (individuals per kg of soil) in all rootstock:variety assemblies. (A) ANOVA parameters for $\log_{10}(x+1)$ transformed nematode numbers. (B) Mean comparison with the LSD Fischer test on transformed nematode numbers at $p>0.05$.

(A)

Source	DoF	Sum of squares	Mean of squares	F	Pr > F
Model	4	0,104	0,026	5,026	0,002
Error	58	0,299	0,005		
Total	62	0,402			

(B)

Modality	Mean	Groups	
SO4: Cs	0,093	A	
Borner: Cs	0,045	A	B
Ném: Cs	0,006		B
Ném: Cal	0,000		B
140Ru: Cal	0,000		B

Spatial distribution. The spatial distribution of *X. index* roughly fitted the distribution of the virus GFLV. No nematodes were recovered from the first two rows in which GFLV- positive or dead plants were the lowest (51% and 29%, respectively). *Xiphinema index* were only recovered in the rows 3 to 5 (CS part) that had the highest numbers of GFLV-positive or dead plants (79% and 97%, respectively) (Fig. 3.18). Our overall data confirmed that the field spatial distribution pattern of *X. index* was aggregative as shown by Villate et al. (2008).

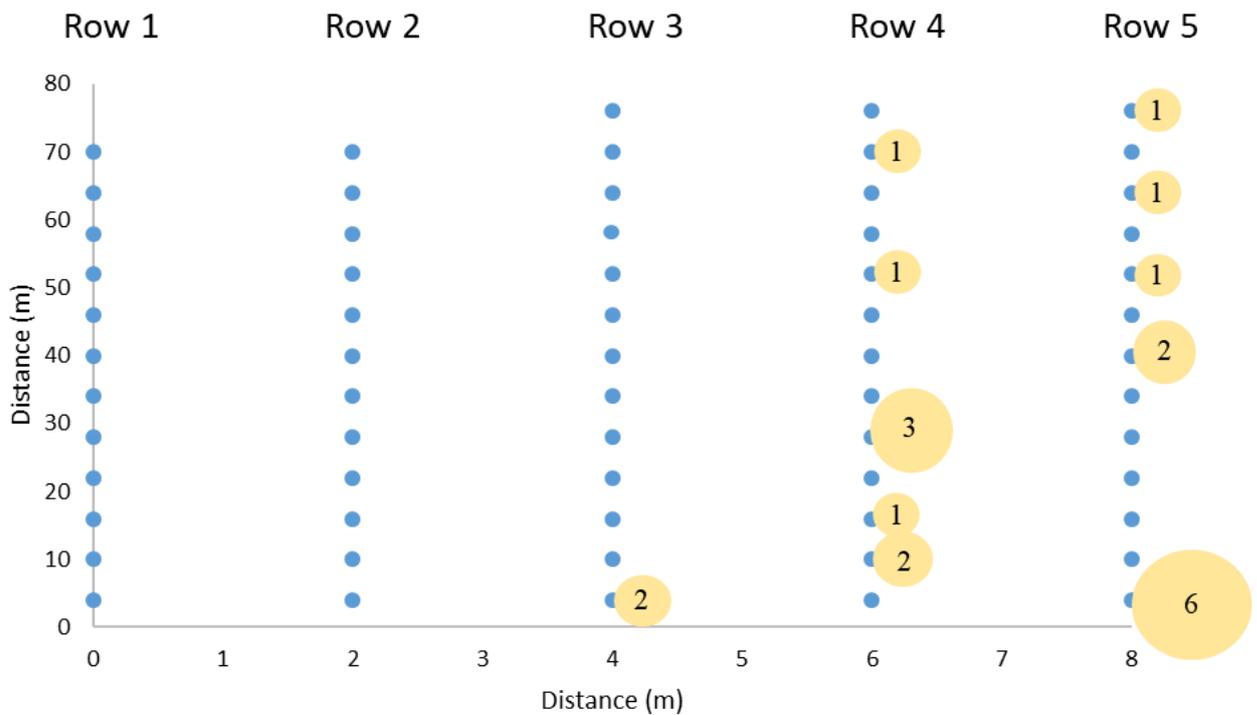


Figure 3.18. Spatial distribution, along the 5 grapevine rows, of the individuals of *X. index* recovered from the field experimental design of Domaine du Chapitre. Blue dots indicate the sampling points and yellow circles give the nematode numbers (per 4 kg of soil) in positive samples.

Detection of *X. pachtaicum*

Nematode numbers. We got a total of 389 *X. pachtaicum* individuals (223 individuals from the first 2-kg sub-sample and 166 individuals from the second 2-kg sub-sample). From the 63 total samples, this represents 6.17 individuals per sample, i.e. a 18-times higher density than *X. index*. Numbers ranged from none to 35 individuals per sample (Fig. 3.19). Nematode numbers were equivalent in each of the three rootstocks grafted with CS and no significant differences between rootstocks could be evidenced after data transformation (Table 3.8). Though 140Ru grafted with Caladoc had lower nematode numbers (presumably due to lower initial numbers in this part of the field), an analysis conducted with all rootstock:variety assemblies only showed significant differences between 140Ru:Cal and the others assemblies except Nem:Cal (Table 3.9).

Table 3.8. ANOVA analysis of *X. pachtaicum* numbers in the rootstocks Börner, Nematex AB and SO4 grafted with Cabernet-Sauvignon. (A) ANOVA parameters for transformed ($\log_{10}x+1$) nematodes numbers. (B) Mean comparison with the LSD Fischer test on transformed nematode numbers at $p>0.05$.

(A)

Source	DoF	Sum of squares	Mean of squares	F	Pr > F
Model	2	0,035	0,017	0,251	0,780
Error	36	2,488	0,069		
Total	38	2,523			

(B)

Modality	Mean	Groups
Ném: Cs	0,463	A
Borner: Cs	0,424	A
SO4: Cs	0,399	A

Table 3.9. ANOVA analysis of *X. pachtaicum* numbers in all rootstock:variety assemblies. (A) ANOVA parameters for transformed ($\log_{10}x+1$) nematodes numbers. (B) Mean comparison with the LSD Fischer test on transformed nematode numbers at $p>0.05$.

(A)

Source	DoF	Sum of squares	Mean of squares	F	Pr > F
Model	4	0,609	0,152	2,539	0,049
Error	58	3,478	0,060		
Total	62	4,086			

(B)

Modality	Mean	Groups	
Ném: Cs	0,463	A	
Borner: Cs	0,424	A	
SO4: Cs	0,399	A	
Ném: Cal	0,356	A	B
140Ru: Cal	0,178		B

Spatial distribution. The species *X. pachtaicum* showed an aggregative pattern of distribution. Nevertheless, individuals were more uniformly dispersed in the field than those of *X. index*. As for this latter species, rows 3 (79 individuals) and 5 (134 individuals) harbored the most numerous focuses but other focuses were also recovered in rows 2 (67 individuals) and 4 (71 individuals). The lowest numbers were in row 1 with only 38 individuals (Fig. 3.19).

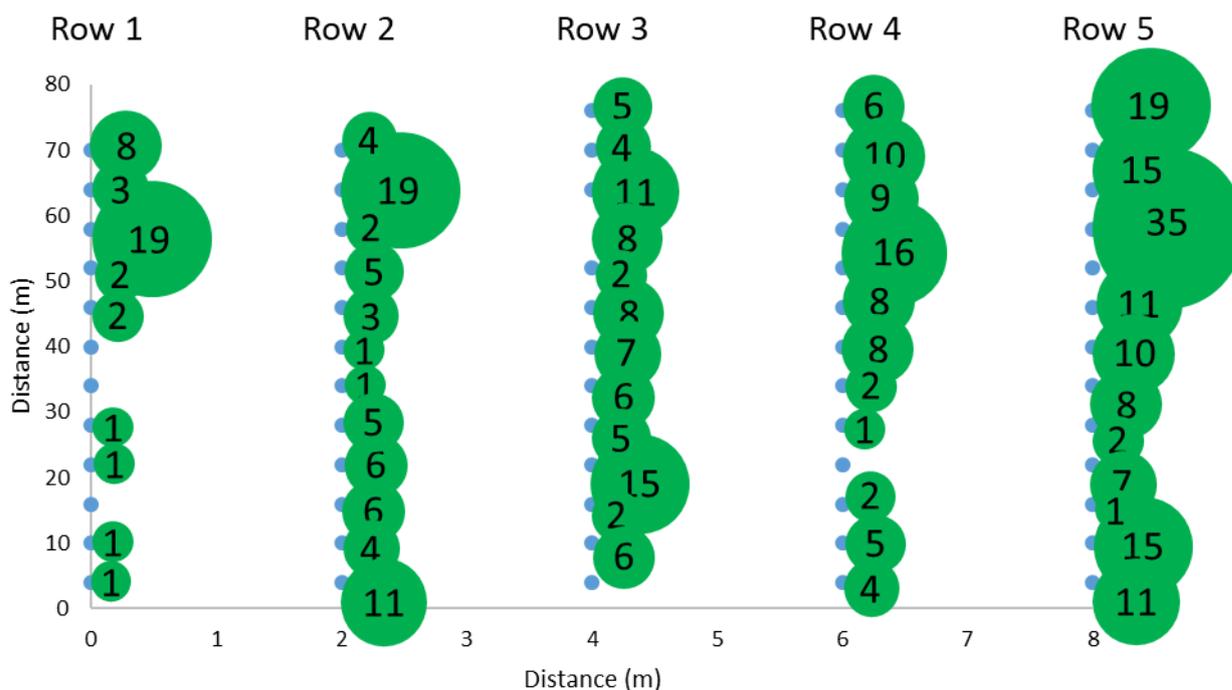


Figure 3.19. Spatial distribution, along the grapevine rows, of the individuals of *X. pachtaicum* recovered from the field experimental design of Domaine du Chapitre. Blue dots indicate the sampling points and green circles give the nematode numbers (per 4 kg of soil) in positive samples.

III.2.5. Discussion and conclusion

III.2.5.1. Discussion

Experimental designs, that aim at studying GFLV control in the field, are classically based on plant development using aerial rating criteria such as pruning and/or bunch weights and on viral symptoms using ELISA from leaf samplings. Because they are costly and labor-intensive, nematode extractions are rarely performed and data about the dynamics of the vector nematode in GFLV control experiments are lacking. In this context, our study, based on a very dense field soil sampling, brings useful complementary data on nematode numbers in the experimental design.

The field plot in which the experiment has been settled had a very high soil GFLV infectious potential. Consequently, as illustrated in Fig. 3.18, the assay has shown early and marked GFLV attacks. Results have clearly illustrated the interest of the rootstock Nemadex AB to delay viral occurrence and symptoms. This experiment is hence considered as the proof of concept of the strategy based on GFLV control through resistance to its vector nematode.

Our results have first confirmed that the spatial distributions of the virus and the nematode are closely related. We illustrated in particular that rows 4 and 5, the most affected by the virus, harbor the highest *X. index* numbers. Very few or no nematodes were recovered

in the first rows, which is in line with the lowest GFLV percentage in this area. The few individuals from *X. index* have only been recovered in the three rows from the CS part of the assay. With the exception of a single individual in one Nemadex AB replicate, individuals of *X. index* were detected in replicates from SO4 and Borner (Fig. 3.19).

We have shown that *X. index* nematode densities were very low 16 years after planting. Such low numbers on that plant age may be essentially due to contrasting host suitability among grapevine rootstocks. On the one hand, plants grafted onto SO4 or 140Ru are either very weak, or hardly surviving or dead because of their susceptibility to nematode and to GFLV attacks and, hence, their limited rooting is expected to have led to the decrease of nematode numbers. On the other hand, the rootstock Nemadex AB has also limited nematode numbers that we can attribute to its resistance behavior. The rootstock Borner expressed as expected an intermediate behavior. Statistical data show that nematode numbers, even though they are low, are significantly higher in nematode (and GFLV) susceptible rootstocks SO4 and 140Ru than in the resistant rootstock Nemadex AB in which almost none has been detected. This strongly suggests that Nemadex AB plants, which are highly vigorous in comparison to other rootstocks, do not harbor nematodes and, consequently, that virulent individuals have not adapted to its resistance factors over years.

By contrast, the more common and non viruliferous species *X. pachtaicum* had much higher numbers and a more dispersed field distribution than *X. index*. From our data, it is difficult to compare and conclude on the host suitability of *X. pachtaicum* to the different rootstocks given that they have very variable vigor and cognate root development. Nevertheless, in our experiment, resistance factors in Nemadex AB did not seem to express on *X. pachtaicum* an effect as marked as on *X. index*.

III.2.5.2. Conclusion

Our study illustrated that nematode densities after 16 years were dependent from both the initial nematode distribution in the field and the rootstock effect. Given that the ability of the nematodes to disperse actively in the soil is extremely limited, we confirmed that the spatial distribution of the vector nematode in the soil and of the aerial virus on the grapevine plants are closely related.

Soil transport allows the diffusion of the virus through its vector. As active dispersal of the nematode *per se* is low, introduction in a new field is most often performed by man. In our experimental field, a single or several introductions may have been performed by soil transport in the entry of rows 4 and 5, from where secondary focuses have passively appeared further along the rows by mechanical soil tillage. This dynamic of the soil infection fits with the data described in Bordeaux vineyards (Villate et al. 2008).

The rare detection of *X. index* in the soil from the resistant accession Nemadex AB strongly suggests that the initial nematode population has not been able to adapt to the resistance factors carried by this rootstock. Even though we have recovered higher numbers of the ubiquitous and quickly colonizing species *X. pachtaicum*, our results did not allow to state whether Nemadex AB also expresses resistance to this nematode. Our results also support the hypothesis that no adaptation of *X. index* to Nemadex AB resistance factors has occurred in the conditions of the field experiment.

The few individuals of *X. index* recovered have not been genotyped individually for their multilocus genotype, but we have characterized other individuals from this field population in the manuscript on phylogeography. These individuals belong to the W1 mitochondrial haplotype (Table S1- Sample #8 designated Xi-Fr-VM from Villeneuve-les-Maguelone, Languedoc, France; Cyt B sequence registered as LT996608).

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CHAPTER 4

CONCLUSIONS AND PERSPECTIVES

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CHAPTER 4: CONCLUSIONS AND PERSPECTIVES

Our works have relied on a phylogeographical approach of the nematode vector *X. index* and on an experimental approach based on plant resistance. Our results have contributed to the global knowledge of *X. index* diversity and to an increased knowledge of its specific behavior in response to the plant resistance pressure. Hence, nematode studies have been conducted at different scales: i) the worldwide scale (phylogeography), ii) the field scale (evolution in a plot from Domaine du Chapitre), and iii) the greenhouse scale, i.e. under controlled conditions (dynamics and genetics). This combination of approaches and scales lead us to an enhanced understanding of several biological and genetic features of *X. index*. The species *X. index*, despite its high economical impact, is poorly studied in particular because of its ectoparasitic status and of its parasitism affecting a perennial crop. In this last chapter, the main results and conclusions from previous chapters will be reminded and discussed. We will then give their implications and perspectives in both the applied agronomic and cognitive research domains.

IV.1. CONCLUSIONS

IV.1.1. The global genetic diversity of *X. index*

To decipher how is shaped the worldwide genetic diversity of *X. index*, we have conducted a study with the highest number ever reported of worldwide geographic samples of the nematode. Our results have leant on both collection populations kept in the greenhouse for variable durations and new samples directly obtained from the field.

The mainly meiotic parthenogenetic species X. index has a lower diversity than the amphimictic species X. diversicaudatum

We first used *X. diversicaudatum*, a sexually-reproducing species, as the amphimictic reference in our study. Mitochondrial data based on the classical *CytB* gene allowed to confirm that the diversity of a mainly parthenogenetic species such as *X. index* is lower than that of its amphimictic sister vector species *X. diversicaudatum*. As the low diversity evidenced with this single gene jeopardized our objective of a reliable picture of the variability of *X. index*, we have used three additional mitochondrial genes. In particular, the *ATP6* gene sequence was shown to be the most polymorphic and thus to bring interesting complementary information. Thus, data based on four mitochondrial genes, generating an unprecedented concatenated sequence of 3044 bp, permitted to draw reliably a basic diversity picture of *X. index*. Our wide array of geographic samples illustrated that *X. index* can be separated into four subclades with well supported nodes.

Both mitochondrial and microsatellite data allow to refine the diversity of X.

index

As a complementary tool to mitochondrial markers, we have used eight microsatellite loci (Villate et al., 2009). Even though the 25 to 30 individuals per population needed for microsatellite population genetics study (Hale et al., 2012) were not available and prevented us from analyzing criteria such as allelic richness, we could successfully refine the low diversity of *X. index* using the few individuals available. Mitochondrial data from ‘samples’ (instead of ‘populations’) permitted us to trace the hypothetical dissemination routes of *X. index* and to reveal that they were closely related to the dissemination routes of its domesticated grapevine host plant. In particular, using microsatellite diversity has given us the complementary ability to refine our hypotheses on the epoch of the major nematode dissemination events.

Nematode lines used in the study are globally representative of the diversity of

X. index

Several lines had been created in 2011 using preliminary data from mitochondrial diversity (Villate et al., 2008). Our results have confirmed these informations and shown that the four lines selected to challenge the resistance can be considered as globally representative of the nematode diversity. Out of them, one (‘Spain’ obtained from the sample Xi-Sp-LM) belongs to the Western (W) subclade, another one (‘Iran’ obtained from the sample Xi-Ir-Sh) belongs to the Near- and Middle-East (NME) subclade while the two others (‘Samos’ obtained from the sample Xi-Gr-Sa, and ‘Italy’ obtained from the sample Xi-It-PC) belong to the Samos and South Italy (SSI) subclade. These latter lines, though they belong to the same subclade, are quite different by their microsatellite pattern, which justified their retention as traceable lines. Interestingly, geographical coverage of the four lines spans widely distant countries highly affected by GFLV and ranging from Middle East (Iran), to Near-East (Samos), up-to the Western Mediterranean major countries, Italy and Spain.

IV.1.2. Plant resistance and nematode adaptation

One major objective was to answer the question whether the nematode *X. index* adapts (and how it may adapt) to plant resistance over years. Given that preliminary data, using plant material obtained from *in vitro*, suggested a progressive adaptation of the nematode to plant resistance, our work aimed first at confirming this putative adaptation. Consequently, we have performed in parallel a comparison of plant material obtained from *in vitro* with classically propagated plant material, i.e. hardwood cuttings rooted into a sandy soil substrate. Our results have brought major information on the effect of the two types of plant propagation on i) the plant resistance and ii) the nematode adaptation.

The mode of plant propagation affects plant resistance

We have studied the effect of the mode of plant propagation on plant resistance with two criteria related to the nematode population dynamics: reproduction factor and population structure.

Using four mixed representative lines, we have first monitored, regardless of the line identities, the evolution of the nematode reproduction factor (RF) in plants aged 3 to 6 years. For both F1 and BC1 resistant material, we could establish that materials obtained from *in vitro* have significantly higher RFs than materials obtained from cuttings. Cuttings expressed a resistance that was durable as shown by their RFs always lower than 1, whatever the plant age. The hypothesis of a 'susceptibilization' effect of *in vitro* culture that preceded our experiment is highly plausible. This effect might be also partly due to IBA, the auxin compound used in the rooting medium just before acclimatization. Nevertheless no IBA effect has been shown on resistant *Prunus* crops towards root-knot nematodes (Esmenjaud et al., 1993). An effect of cytokinins, as observed in tomato (*S. lycopersicum*, *S. sisymbriifolium*) (Dropkin et al., 1969) or *Musa* spp. (Elsen et al., 2002) towards *Meloidogyne* and *Radopholus similis*, respectively, is also excluded as multiplication and rooting culture media were free of these hormones. Nematode progressive multiplication in R accessions obtained from *in vitro* appear linked to a different architecture of the root system in this propagation type that may have induced discrete but putatively durable physiological changes in apical root tissues from where nematodes feed. The effect of *in vitro* culture has already been evidenced in interactions between plants and endoparasitic nematodes, but it is reported for the first time here for an ectoparasitic nematode species. Furthermore, the unprecedented duration of 'at least 6 years' suggests that the plant has an unsuspected long memory of its *in vitro* past. For *R. similis* in *Musa* spp. for example, the effect has been shown to be temporary (8 weeks) (Elsen et al., 2002).

We could also highlight that, besides harboring contrasting nematode numbers, cutting and *in vitro* resistant materials exhibit different population structures, after 5 and 6 years, characterized by a number of juveniles significantly lower in cutting plants than in *in vitro* plants. In resistant accessions, this phenomenon logically illustrates the lower reproductive potential of females from cutting plants in comparison with plants obtained from *in vitro*. Females and 4th-stage juveniles have been shown to survive under starving conditions at stable temperatures of 7 and 20°C for over 4 years (Demangeat et al., 2005). This duration might be the maximal limit for juveniles, but females might have the ability to survive longer, i.e. up-to 6-7 years. Our results for resistant cutting material confirm that the 'eradication' of the nematodes, and mainly of the females, is particularly difficult: it illustrates the need for long duration experiments in studies on population dynamics of *X. index* faced to resistant accessions as well as to other control methods such as fallow crops.

The differences between *in vitro* and cutting plant material observed above in the ratio juveniles/adults were not seen in the ratio males/females. Even though the recognition of the rare males among the high numbers of females is not easy and may have generated poorly reliable data, we did not get any evidence that the sex ratio was influenced by the propagation type or the resistance status of the plant. Consequently, our results do not bring conclusive data in these domains. By contrast, in RKNs, high nematode densities or lack of food resources (Davide & Triantaphyllou, 1967) or the presence of resistance genes in the host (Moura et al., 1993) are known to induce the development of an increased rate of males.

Despite the evidence of sexual reproduction, its involvement in the adaptation of the nematode is poorly plausible

The putative adaptation of *X. index* has been studied first with a nematode genetics approach. Our results have confirmed that microsatellites are reliable markers for monitoring and/or tracing nematode line evolution. Characterization of microsatellite MLGs permitted us to detect a few but clearly identified hybrid individuals between different lines at increasing rates between 4 and 6 years. Thus, our results did confirm under controlled conditions the ability of *X. index* to reproduce sexually as already observed in the vineyard (Villate et al., 2010).

The occurrence of hybrids in plants was detected from both types of plant propagation. Our results thus suggest that the nematode may alternate sexual or asexual reproduction modes, even though sexual reproduction appears rare and independent of its biotic environment (R or S accession). This supports the hypothesis that the 'decreased' resistance observed from resistant *in vitro* material is not due to an adaptation of the nematode consecutive to hybridization. To go further, this also reinforces the probability that we have evidenced an apparent but not real resistance 'overcoming', a phenomenon that is independent from the nematode and might be due to a modified physiological status of the plant. However, our results do not discard totally the possibility that nematodes reared on resistant material obtained from *in vitro* may progressively acquire adaptative (or virulence?) factors during their long interaction with the plant. Nevertheless, these results, by showing that the resistance from cutting material is durable (over 6 years), conversely suggest that such factors are not selected/expressed under the continuous resistance pressure exerted by the plants from this propagation type. Out of the four lines used and whatever the resistance status of the plant material, our results show differences in lines aggressiveness. Nevertheless, these differences did not induce a total elimination or conversely a complete selection of one of the lines over years. Hence, it appears that, despite their variable aggressiveness, only Iran (and eventually Italy) might have undergone the selection of (a) major adaptative factor(s). This also illustrates the absence of a marked genetic drift between populations kept in the ISA nematode

collection (notably between the population 'Italy' introduced in 1993 in the collection of UMR ISA and the others that date back from 2005-2006, i.e. 12 years later).

Muscadine-derived material appears durably resistant

The adaptation of the nematode to the plant resistance has not been observed on F1 and BC1 materials obtained from classical hardwood cutting propagation in our experimental data under controlled conditions over 6 years. The field study dealing with the nematode distribution in a 16-year old experiment has brought complementary information that are in line with that of the greenhouse experiment as nematodes also did not appear to be able to develop on the BC1 rootstock RPG1 (Nemadex AB). Thus, the control of the nematode vector by a resistance strategy based on muscadine-derived material appears durable.

IV.2. PERSPECTIVES

IV.2.1. The global genetic diversity of *X. index* still needs to be refined

Even though our study has benefitted from a wide range of samples, our sampling is far from exhaustive. Many gaps need to be fulfilled to complete the dissemination view. These are mainly: i) Eastern Mediterranean uncovered areas (Syria, Iraq, Eastern Turkey, Azerbaijan, Georgia, Armenia...), ii) Northern Africa (Egypt, Tunisia, Algeria, Morocco...), iii) Andean countries other than Chile and Argentina (Peru, Ecuador, Colombia...), and iv) Western European dissemination areas from both the Antiquity and the Modern Era (*Phylloxera* crisis).

The Eastern Mediterranean harbors the highest diversity and our few geographical sampling points only give a preliminary picture of this region that needs to be completed by a more reliable coverage. These new elements might be obtained in the near future through close collaborations with local researchers in agronomy together with Archaeologists or specialists more particularly interested in Archaeobotanics. These links would be particularly appreciated to decipher i) the Phoenician heritage and its putative routes from Lebanon and ii) the hypothetical initial focus(es) of Western introductions of grape by the Greco-Romans.

We also lack data from Northern Africa that could show whether a specific introduction and dissemination route by the Phoenicians and/or later by the Arabs exists in Southern Mediterranean countries. We do know that the nematode is present in Maghreb countries (Dalmaso & Cuany, 1969; Siddiqi, 1986) in areas that had been planted with grapevine by the French in the 19th and 20th centuries after the European colonization (A. Dalmaso, A. Cuany and C. Scotto La Massèse, pers. com.) and consequently at least populations from the Western haplotype should be detected there.

In Southern America and in particular in Andean countries, a survey for new populations would be very useful to bring additional information on the apparent marked bottleneck effect

that we revealed in Chile and Argentina. Our study could benefit from a deeper investigation dealing with the historical routes of the conquest of South America, and of the Andean regions in particular, by the Spanish colons. In Peru, Bolivia, Ecuador and Columbia, these data might detect introduction events that would be independent from those revealed in the two more Southern Andean countries that we have explored.

In Western European countries, except in the Southern Iberian Peninsula and in the Bordeaux vineyards, we lack samples from European regions in which initial introduction may be attributed to Romans. In Southern France notably, the single haplotype from Fréjus that we have assigned to this late-Antiquity Epoch is certainly not isolated and complementary informative data would be greatly valuable. Another interesting research field could be dedicated to the dispersal of nematode-contaminated plant material consecutive to the phylloxera crisis at the end of the 19th century. Because of their still recent historical occurrence, these events should be easier to document. This might be a good example to illustrate how a soil-dwelling nematode with a very limited self-mobility and few host plants has become a parasite with a high economical impact. Grapevine has great cultural and societal impacts, notably in Southern European countries. Thus, in the reverse sense, reconstituting the putative initial focus(es) and dissemination route(s) of the resistant grapevine rootstocks selected and diffused at that time, using *X. index* as a marker, would be quite original. Such data will contribute to understand the putative and unexpectedly quick dispersal of the nematode at this time and its today's major involvement in GFLV-linked grapevine decline (i.e. current 'dépérissement de la vigne'), particularly in France.

IV.2.2. Diversity data (and nematode lines) will be a baseline for evaluation of control methods

Besides rootstock resistance to *X. index*, current research against GFLV includes another major approach based on the use of fallow crops with an antagonistic effect against the vector nematode. In this objective, ongoing experiments conducted in France all deal with populations from the Western (W) subclade. A pioneer work, reporting notably the antagonistic effect of hairy vetch, in an experimental field from the Bordeaux vineyard (Villate et al., 2013) has also considered a population that belongs to this subclade. Genetically-distant populations may show different behaviors to antagonistic plants: extending experimental results to countries harboring other *X. index* subclades would need to take into account populations from these subclades.

IV.2.3. Plant resistance and nematode adaptation

Deciphering how the mode of plant propagation affects plant resistance is a challenging task

Our data do not permit to know how long the ‘suceptibilization’ phenomenon associated to the *in vitro* origin of the resistant material may last, beyond the 6 years of our study. As grapevine plants are perennial crops grown for more than 20-30 years, it is highly plausible but not sure that they gradually recover their full resistance. The link between the architecture of the root system in this propagation type and the discrete but putatively durable physiological changes in apical root tissues from where nematodes feed merits further investigations. These changes are advantageously exploited by the ectoparasitic nematode to facilitate its feeding and subsequent reproduction. Deciphering how an *in vitro* plant environment may induce putatively durable changes underlying the ‘susceptible’ physiological status expressed by the plant is a challenge.

Our results highlight the need for a better knowledge of the histological mechanisms of resistance to *X. index*. In a susceptible plant, the nematode triggers a (limited) cell polyploidization and a cognate feeding site. In muscadine-derived resistant plants, studies on the histological mechanisms of resistance show that a hypersensitive response is induced in both *in vitro* and cuttings plant materials (D. Esmenjaud and M. Y. Banora, unpublished data). Our data on the dependence of resistance on the type of plant multiplication suggest that, after an initial *in vitro* propagation step, the cell hypersensitive response might be delayed in a manner that is apparently incipient for the nematode but that would allow its slightly prolonged feeding from apical root tissues. This ‘suceptibilization’ may act by gently but lastingly ‘softening’ the cell wall structure and thus by delaying the critical point from where the feeding cells will trigger a hypersensitive response.

Is there indeed an adaptation of the nematode having faced resistance in plants obtained from in vitro?

A definitive proof of the absence of a specific adaptation of the nematode consecutive to its inoculation on resistant material obtained from *in vitro* is still needed. It might be performed, on a resistant accession obtained from cuttings, by comparing the population dynamics of i) ‘(pre)adapted’ individuals obtained from *in vitro* on this accession (carrying the R factors) and ii) ‘non (pre)adapted’ individuals obtained from a susceptible grapevine accession (lacking the R factors). We cannot exclude that nematodes that have faced a ‘softened’ resistance have acquired (selected) some pathogenicity factors that might facilitate their subsequent ability to overcome a non-modified (‘natural’) resistance, represented in our study by plants obtained from cuttings.

What are the determinants driving or influencing the nematode shift from a reproduction mode to another?

We still ignore in *X. index* the determinants of the change from a reproduction mode to another. These factors may be either biotic or/and abiotic. Studies aiming at identifying them appear necessary but very difficult to conceive. Our study failed to reveal that the plant resistance selection pressure was a major determinant. According to McDonald and Linde (2002), pests with a high evolutionary potential have a higher ability to overcome plant resistance. Thus, pest populations that show the highest probability to overcome resistance are those with a dual reproduction mode. Even though sexual reproduction should contribute to the adaptation of *X. index* to diverse biotic or abiotic environments, our results did not bring evidence of its involvement to face plant resistance.

Muscadine-derived material is a promising strategy to contribute to GFLV control

Durability is closely linked to the genetic determinism of resistance. Our results highlight the need for a better knowledge of the genetics of resistance to *X. index* in muscadine-derived material. Ongoing research suggests that resistance is conferred by several independent factors that would be major genes or QTLs. Nevertheless, the availability of a progeny of less than hundred individuals from the BC1 segregating cross between the distant *Vitis* and *Muscadinia* genera compromise the precise mapping of resistance. However, the durable resistance, revealed after 6 years under controlled conditions and highly suggested after 16 years under field conditions, might be related to this polygenic status. The creation of monogenic BC2 progenies, i.e. carrying a single putative gene, is currently attempted at UMR EGFV. The subsequent evaluation of these BC2 progenies at UMR ISA would allow to confirm the polygenic status of resistance. Such material will also open new opportunities to relate genetic, histological and durability data.

Arresting or delaying GFLV transmission to grapevine by acting on the vector nematode through plant genetic resistance is a promising alternative. The successful control of the vector nematode, by reducing or delaying GFLV transmission in the field, should be a major component of an integrated control of the virus. Our results bring information that will be useful for the grapevine community and in particular for grapevine breeders, nurserists and growers.

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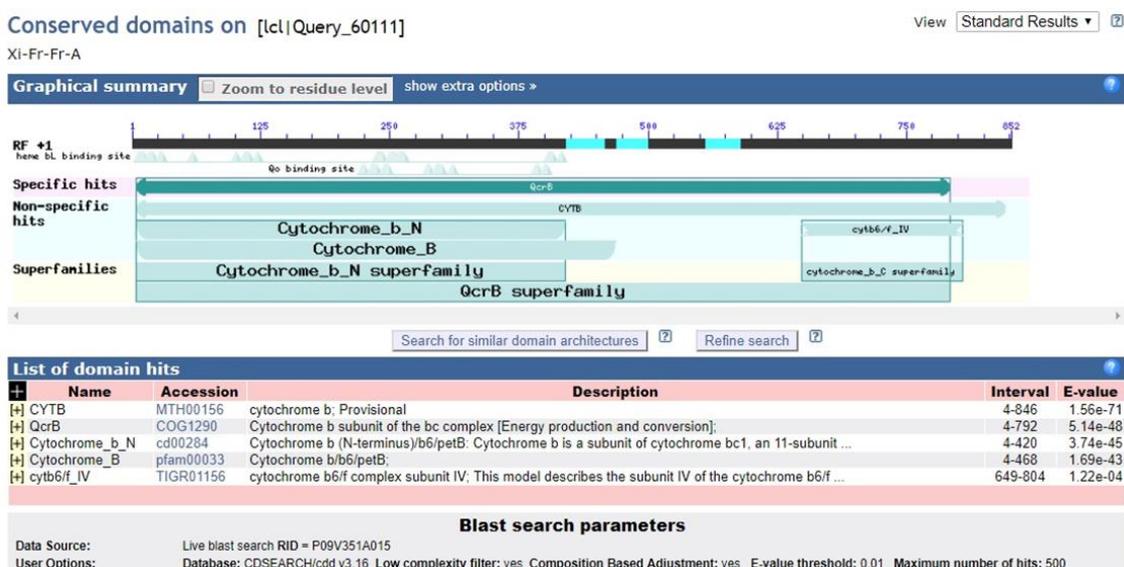
ANNEXES

Supporting information – Chapter 2

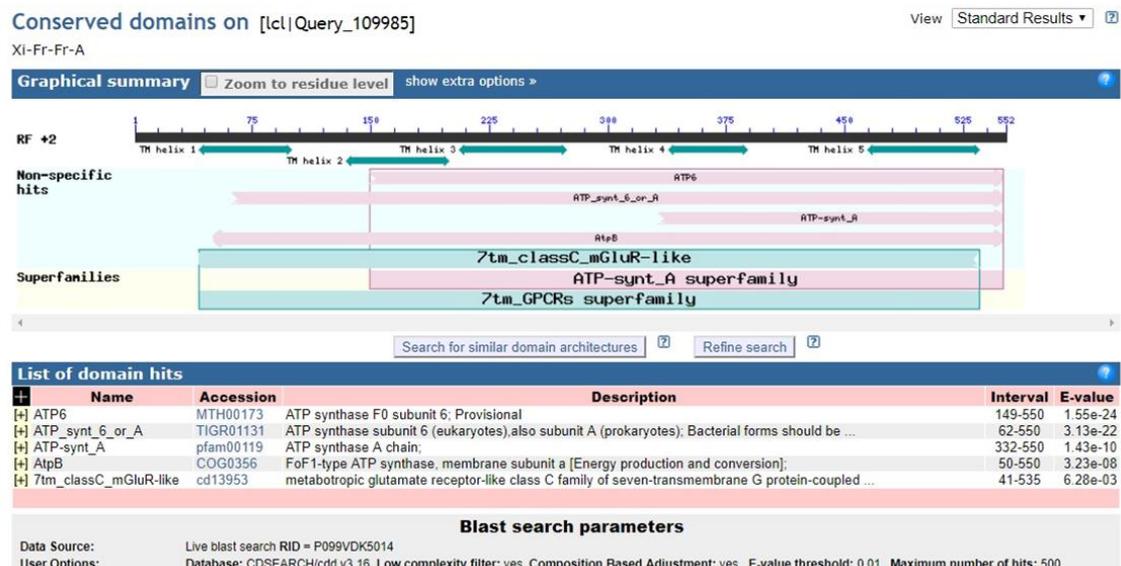
These annexes concern the four mitochondrial genes used in our study. We first illustrate that the sequences used contain the functional domains of each gene (Fig. S3). The representative haplotype analyzed is Xi-Fr-Fr-A. Then we report the complete 3044-bp sequence obtained from the four concatenated genes (Fig. S4) for a set of 10 representative haplotypes selected from Fig. 2A in the manuscript.

Conserved domains of the four mitochondrial genes *CytB*, *ATP6*, *CO1* and *ND4*

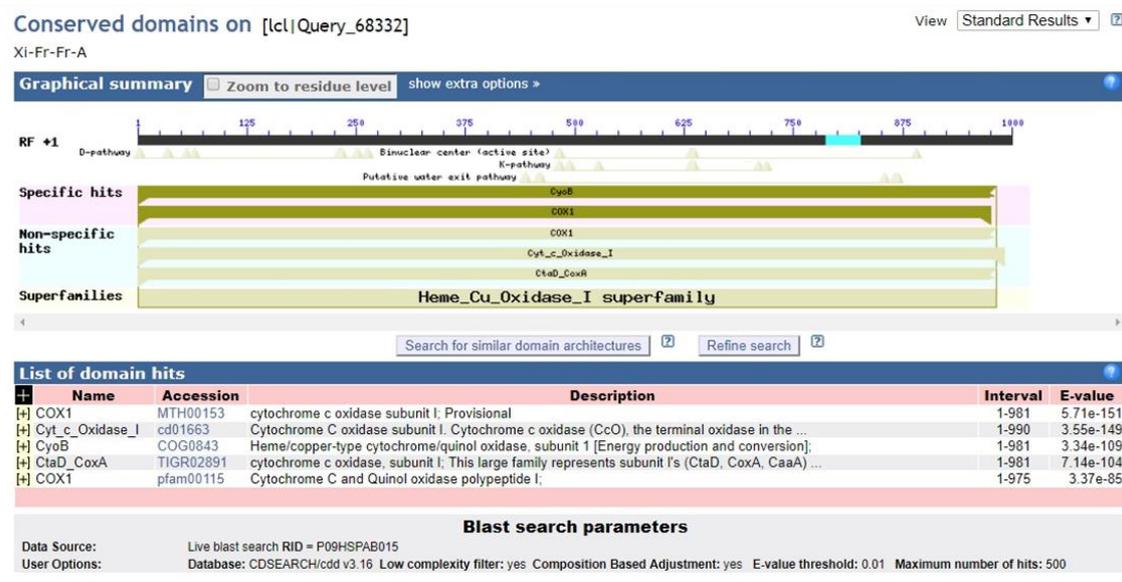
A



B



C



D

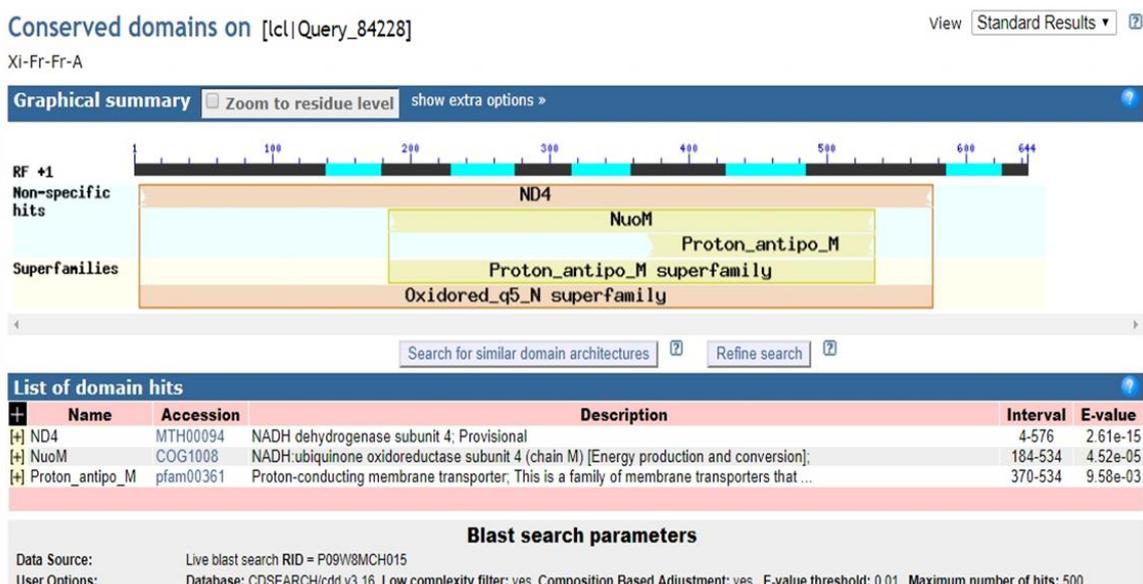


Figure S3. Conserved domains of the four mitochondrial genes *CytB* (A), *ATP6* (B), *CO1* (C) and *ND4* (D) using BLASTX (nucleotide → protein). The reference genetic code is 'Invertebrate mitochondrial'. For the *CytB* and *CO1* genes, as there were several close species sequences available on the database, our sequences matched specific *CytB* and *CO1* domains and revealed these respective superfamilies. For *ATP6* and *ND4*, only non-specific hits were detected, which is due to the absence of other sequences of these nematodes in the database. Nevertheless, our sequences did belong to the superfamilies *ATP* and *ND4*, respectively.

Figure S4. Multiple sequence alignment (CLUSTAL 2.1) of the complete 3044-bp sequence obtained from the four concatenated mitochondrial genes (*CytB*, *ATP6*, *CO1* and *ND4*). A set of 10 representative haplotypes selected from Fig. 2A in the manuscript is used. For the codes, see Table S1

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Xi-Fr-SE1      AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Fr-Mel1    AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Ir-Ta-A    AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Ir-Ta-B    AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Ir-Sh      AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Eg-Is     AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Tu-Al-B   AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-Gr-Sa-AB  AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-It-PC     AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
Xi-CJ-Je     AGCTCAGGCATAATTCAGCCTTATATTATGTAAGAGGTAAGTACTAGAGCATGGGATTCAGTA
*****

Xi-Fr-SE1      GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Fr-Mel1    GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Ir-Ta-A    GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Ir-Ta-B    GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Ir-Sh      GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Eg-Is     GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Tu-Al-B   GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-Gr-Sa-AB  GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-It-PC     GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
Xi-CJ-Je     GTAGAAATAACACGAGAAGTATATGCAGGATGAATAATTCGTTCAATTCATAGTAACACA
*****

Xi-Fr-SE1      GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Fr-Mel1    GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Ir-Ta-A    GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Ir-Ta-B    GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Ir-Sh      GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Eg-Is     GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Tu-Al-B   GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-Gr-Sa-AB  GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-It-PC     GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
Xi-CJ-Je     GCATCTTTTGT TTTTATTATCTCTTTTACATTTTCTTCGGGGAATATCACAAAGCCTCT
*****

Xi-Fr-SE1      TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Fr-Mel1    TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Ir-Ta-A    TTTTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Ir-Ta-B    TTTTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Ir-Sh      TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Eg-Is     TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Tu-Al-B   TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-Gr-Sa-AB  TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-It-PC     TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
Xi-CJ-Je     TTCTACTTAAAAGGACCCCTGAATAAGAGGATGAACAATTATATTATTAACATATGGCTGCT
*****

Xi-Fr-SE1      GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Fr-Mel1    GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Ir-Ta-A    GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Ir-Ta-B    GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Ir-Sh      GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Eg-Is     GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Tu-Al-B   GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-Gr-Sa-AB  GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-It-PC     GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
Xi-CJ-Je     GCCTTTCTAGGATATGTATTACCTTGAGGTCAAATGTCATTTTGAGGAGCTACTGTTATT
*****

Xi-Fr-SE1      ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-Fr-Mel1    ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-Ir-Ta-A    ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-Ir-Ta-B    ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-Ir-Sh      ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-Eg-Is     ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
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Xi-Gr-Sa-AB  ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-It-PC     ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
Xi-CJ-Je     ATTAATCTACTCAGCGTAATACCAATAGGAAAACTTTAGTCACTTGACTATGAGGGGGA
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Xi-Fr-SE1      TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
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Xi-Ir-Ta-A    TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
Xi-Ir-Ta-B    TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
Xi-Ir-Sh      TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
Xi-Eg-Is     TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
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Xi-It-PC     TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
Xi-CJ-Je     TTTTATGTGTCGCGCTTTTACTTGTAGATTCTTTTTTGCTCTTCATTTTATTTTGCCCTTTT
*****

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Xi-It-PC     GGAGTATTAGGAGGAGGAGCAATTCATTTATTATTACTTCATAAAAACGGGAAGAAGGAGA
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*****

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Xi-It-PC     TTTAGAGGTAGAAGAACTCTCATGCTCTAAAAATTAATTTAGACACTTATTTTCTTAT
Xi-CJ-Je     TTTAGAGGTAGAAGAACTCTCATGCTCTAAAAATTAATTTAGACACTTATTTTCTTAT
*****

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Xi-CJ-Je     ATTCAGCCAGAATGGTACTTCTTACACTTTTACGCTATCCTACGATCAATTCCAAACAAA
*****

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Xi-Ir-Sh      TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
Xi-Eg-Is     TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
Xi-Tu-Al-B   TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
Xi-Gr-Sa-AB  TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
Xi-It-PC     TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
Xi-CJ-Je     TTGGGAGGATTGGTAGCATTGGACTTGCTTTTCATAGTATTACTAGTACTCAGTATGGTA
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Xi-Fr-SE1      AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Fr-Mel1    AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Ir-Ta-A    AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Ir-Ta-B    AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Ir-Sh      AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Eg-Is      AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Tu-Al-B    AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-Gr-Sa-AB   AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-It-PC      AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
Xi-CJ-Je      AATTCTACACAAAGAGTAGTAAACATTGAATTCCTTTTCATTAATAAACTGAAGATTTATT
*****

Xi-Fr-SE1      AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
Xi-Fr-Mel1    AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
Xi-Ir-Ta-A    AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTCGATTTTGAATATAAAAATTTATTTT
Xi-Ir-Ta-B    AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTCGATTTTGAATATAAAAATTTATTTT
Xi-Ir-Sh      AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
Xi-Eg-Is      AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTCGATTTTGAATATAAAAATTTATTTT
Xi-Tu-Al-B    AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
Xi-Gr-Sa-AB   AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
Xi-It-PC      AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTCGATTTTGAATATAAAAATTTATTTT
Xi-CJ-Je      AGAGTTAATGCCTTTGTTAACTTATTTTGCCCTTTGATTTTGAATATAAAAATTTATTTT
*****

Xi-Fr-SE1      CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Fr-Mel1    CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Ir-Ta-A    CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Ir-Ta-B    CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Ir-Sh      CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Eg-Is      CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Tu-Al-B    CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-Gr-Sa-AB   CGGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-It-PC      CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
Xi-CJ-Je      CAGTGTATTATTATGATAATAACAACCTTAGTTTTTATTTTTTTATTTTGTGAGTTT
* *****

Xi-Fr-SE1      AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Fr-Mel1    AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Ir-Ta-A    AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Ir-Ta-B    AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Ir-Sh      AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Eg-Is      AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Tu-Al-B    AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-Gr-Sa-AB   AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-It-PC      AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
Xi-CJ-Je      AGATAGAAAATTAGTAGGGGTTTTTATGAAAATAACTACGTTTTAAGTATAGGAGCTGT
*****

Xi-Fr-SE1      ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Fr-Mel1    ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Ir-Ta-A    ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Ir-Ta-B    ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Ir-Sh      ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Eg-Is      ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Tu-Al-B    ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-Gr-Sa-AB   ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-It-PC      ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
Xi-CJ-Je      ACTATTAATTTGTTTAAATTAATTTCTTTAGTGTGTTATTCTTATCCAGTAACACTAC
*****

Xi-Fr-SE1      TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Fr-Mel1    TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Ir-Ta-A    TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Ir-Ta-B    TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Ir-Sh      TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Eg-Is      TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Tu-Al-B    TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-Gr-Sa-AB   TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-It-PC      TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
Xi-CJ-Je      TCTCTCCTTTAATTTTAGTGTGCGGTTGTTTTATGAACTCTAGTTTAAATTTTTTATT
*****

Xi-Fr-SE1      TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Fr-Mel1    TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Ir-Ta-A    TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Ir-Ta-B    TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Ir-Sh      TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Eg-Is      TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Tu-Al-B    TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-Gr-Sa-AB   TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-It-PC      TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
Xi-CJ-Je      TTTAAAACCTAAAACCTATGGCTGCTATTTTGCCCTCTAATTCCTCCCTGATACTTGATTCC
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Xi-Fr-SE1      TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Fr-Mel1     TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Ir-Ta-A     TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Ir-Ta-B     TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Ir-Sh       TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Eg-Is       TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Tu-Al-B     TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-Gr-Sa-AB    TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-It-PC       TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
Xi-CJ-Je       TTTTTGTCTATAGTAGAATTAGTAAGCTTATTAGTTCGTCAGTAACCTTTATCTTTTCG
*****

Xi-Fr-SE1      ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Fr-Mel1     ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Ir-Ta-A     ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Ir-Ta-B     ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Ir-Sh       ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Eg-Is       ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Tu-Al-B     ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-Gr-Sa-AB    ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-It-PC       ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
Xi-CJ-Je       ATTGCTAGCCAATATGAGAGCTGGGCATATTTTACTTCTTTAATTTGTAAAAATACCTC
*****

Xi-Fr-SE1      GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Fr-Mel1     GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Ir-Ta-A     GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Ir-Ta-B     GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Ir-Sh       GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Eg-Is       GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Tu-Al-B     GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-Gr-Sa-AB    GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-It-PC       GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
Xi-CJ-Je       GGAATATGATTACTAGGAAGAGTTTTTGGAAATGTTAGAACTTATAGTATGTGTAGTTCA
*****

Xi-Fr-SE1      AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Fr-Mel1     AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Ir-Ta-A     AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Ir-Ta-B     AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Ir-Sh       AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Eg-Is       AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Tu-Al-B     AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-Gr-Sa-AB    AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-It-PC       AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
Xi-CJ-Je       AGCTTTCGTTTTCTCATATTAATGTATTAATTCCTTTAATATTAGGGGCACAAGACAT
*****

Xi-Fr-SE1      AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Fr-Mel1     AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Ir-Ta-A     AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Ir-Ta-B     AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Ir-Sh       AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Eg-Is       AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Tu-Al-B     AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-Gr-Sa-AB    AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-It-PC       AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
Xi-CJ-Je       AAGTTTTCCCGCTCAAATAATTTTAGGTTTGAATTTTGCCTTTTAGAGGAACTTTTAT
*****

Xi-Fr-SE1      TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Fr-Mel1     TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Ir-Ta-A     TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Ir-Ta-B     TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Ir-Sh       TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Eg-Is       TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Tu-Al-B     TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-Gr-Sa-AB    TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-It-PC       TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
Xi-CJ-Je       TATAAGATCCCTGCTTGTTAGAACAGGGGCCGGAACAGGGTGGACTATTTATCCCCCCT
*****

Xi-Fr-SE1      AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Fr-Mel1     AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Ir-Ta-A     AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Ir-Ta-B     AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Ir-Sh       AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Eg-Is       AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Tu-Al-B     AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-Gr-Sa-AB    AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-It-PC       AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
Xi-CJ-Je       AAGAAGAGTTGTAGGTCATTCTGACTTAAGAGTAGACATAGTAATTTTCAGTCTTCACAT
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Xi-Fr-SE1      AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Fr-Mel1    AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Ir-Ta-A    AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Ir-Ta-B    AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Ir-Sh      AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Eg-Is      AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Tu-Al-B    AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-Gr-Sa-AB   AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-It-PC      AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
Xi-CJ-Je      AGCAGGAGTAAGATCTATTGCAGGTTCTATCAATTTTTTATCTACTATTAACAATTTAAA
*****

Xi-Fr-SE1      GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Fr-Mel1    GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Ir-Ta-A    GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Ir-Ta-B    GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Ir-Sh      GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Eg-Is      GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Tu-Al-B    GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-Gr-Sa-AB   GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-It-PC      GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
Xi-CJ-Je      GGGGGAATCGTTAAGATTTCTTAACTTACCATTATTCCTGGTTAGAGTTGGGTAAGTGC
*****

Xi-Fr-SE1      TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Fr-Mel1    TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Ir-Ta-A    TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Ir-Ta-B    TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Ir-Sh      TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Eg-Is      TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Tu-Al-B    TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-Gr-Sa-AB   TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-It-PC      TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
Xi-CJ-Je      TTTTTTATTAGTGTTAAGCTTGCCTGTATTAGCAGGAGGTATTACTATACCTCTTTTTGA
*****

Xi-Fr-SE1      TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Fr-Mel1    TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Ir-Ta-A    TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Ir-Ta-B    TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Ir-Sh      TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Eg-Is      TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Tu-Al-B    TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-Gr-Sa-AB   TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-It-PC      TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
Xi-CJ-Je      TCGAAATTTAATTCCTCTTCTTTGATCCACTAGGAGGCGGGGATCCTTTACTGTTTCA
*****

Xi-Fr-SE1      ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Fr-Mel1    ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Ir-Ta-A    ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Ir-Ta-B    ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Ir-Sh      ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Eg-Is      ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Tu-Al-B    ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-Gr-Sa-AB   ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-It-PC      ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
Xi-CJ-Je      ACATTTATTCTGATTCCTCGGACATCCTGAAGTTTATATTTAATTTTGCCTGGATTG
*****

Xi-Fr-SE1      TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Fr-Mel1    TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Ir-Ta-A    TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Ir-Ta-B    TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Ir-Sh      TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Eg-Is      TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Tu-Al-B    TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-Gr-Sa-AB   TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-It-PC      TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
Xi-CJ-Je      TCTAGTTAGACACGCGGTTATTATTAGAAGTGGAAAAAATCCCCCTTTGGTACCCAGG
*****

Xi-Fr-SE1      CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Fr-Mel1    CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Ir-Ta-A    CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Ir-Ta-B    CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Ir-Sh      CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Eg-Is      CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Tu-Al-B    CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-Gr-Sa-AB   CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-It-PC      CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
Xi-CJ-Je      CATATTTTTAGCTATTGCTAGAATTGGAGTCTTAGGCTGCGTAGTATGAGCACATCATAT
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Xi-Fr-SE1      AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Fr-Mel1    AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Ir-Ta-A    AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Ir-Ta-B    AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Ir-Sh      AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Eg-Is      AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Tu-Al-B    AATAAGAGTAGGACTTGATATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-Gr-Sa-AB  AATAAGAGTAGGACTTGACATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-It-PC      AATAAGAGTAGGACTTGACATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
Xi-CJ-Je      AATAAGAGTAGGACTTGACATAGATACTCGACTATACTTTACAGCCGCCACTATAATCAT
*****

Xi-Fr-SE1      TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Fr-Mel1    TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Ir-Ta-A    TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Ir-Ta-B    TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Ir-Sh      TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Eg-Is      TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Tu-Al-B    TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGCAGTTTTTT
Xi-Gr-Sa-AB  TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGTGTGTTTTTT
Xi-It-PC      TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGTGTGTTTTTT
Xi-CJ-Je      TGCAGTTCCTACAGGCATTAAGTTTTAGGTGAATTGCTTCTCTAAGTGGTGTGTTTTTT
*****

Xi-Fr-SE1      ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Fr-Mel1    ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Ir-Ta-A    ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Ir-Ta-B    ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Ir-Sh      ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Eg-Is      ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Tu-Al-B    ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-Gr-Sa-AB  ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-It-PC      ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
Xi-CJ-Je      ACTAATAAAAACCTTTACAGGCTTGGATTCTAGGATTCCTATTCTTTCCACCATAGGGGG
*****

Xi-Fr-SE1      ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Fr-Mel1    ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Ir-Ta-A    ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Ir-Ta-B    ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Ir-Sh      ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Eg-Is      ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Tu-Al-B    ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-Gr-Sa-AB  ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-It-PC      ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
Xi-CJ-Je      ATTAACAGGAATTGTATTAGCTAATAGAACCCTTAGATTTAATCTATCATGACACTTACTA
*****

Xi-Fr-SE1      TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Fr-Mel1    TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Ir-Ta-A    TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Ir-Ta-B    TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Ir-Sh      TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Eg-Is      TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Tu-Al-B    TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-Gr-Sa-AB  TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-It-PC      TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
Xi-CJ-Je      TGTAGTAGCTCATTTCCTACTACGTTTTAAGTATAGGAGCTGTATTTCGCCATTATAGTTGG
*****

Xi-Fr-SE1      TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCACATAT
Xi-Fr-Mel1    TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCACATAT
Xi-Ir-Ta-A    TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCGCATAT
Xi-Ir-Ta-B    TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCGCATAT
Xi-Ir-Sh      TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCGCATAT
Xi-Eg-Is      TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCGCATAT
Xi-Tu-Al-B    TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCGCATAT
Xi-Gr-Sa-AB  TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCACATAT
Xi-It-PC      TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCACATAT
Xi-CJ-Je      TTTAACTGCATGGTGACCAATAGTAACAGGAACCTTGTCTAATACTATTTTATCACATAT
*****

Xi-Fr-SE1      CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Fr-Mel1    CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Ir-Ta-A    CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Ir-Ta-B    CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Ir-Sh      CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Eg-Is      CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Tu-Al-B    CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-Gr-Sa-AB  CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-It-PC      CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
Xi-CJ-Je      CCGGAACGTTTGTAGTGCTTGTATTATTATTATTATTATACTGTTAGGTTTTCTTTGCCC
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Xi-Fr-SE1      TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Fr-Mel1    TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Ir-Ta-A    TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Ir-Ta-B    TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Ir-Sh      TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Eg-Is     TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Tu-Al-B   TTTATATTTTTTGTGTTATGGCTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-Gr-Sa-AB  TTTATATTTTTTGTGTTATGACTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-It-PC     TTTATATTTTTTGTGTTATGACTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
Xi-CJ-Je     TTTATATTTTTTGTGTTATGACTGTAGGCATAAAATTTATTTTTTTACTCAAAATAAGA
*****

Xi-Fr-SE1      GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Fr-Mel1    GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Ir-Ta-A    GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Ir-Ta-B    GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Ir-Sh      GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Eg-Is     GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Tu-Al-B   GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-Gr-Sa-AB  GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-It-PC     GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
Xi-CJ-Je     GTTAGTATAATATTGTCGGTTTTTTTTGTTTTTCCCTTTTTTTGTAAAGTTACCAGTATTT
*****

Xi-Fr-SE1      GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Fr-Mel1    GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Ir-Ta-A    GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Ir-Ta-B    GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Ir-Sh      GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Eg-Is     GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Tu-Al-B   GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-Gr-Sa-AB  GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-It-PC     GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
Xi-CJ-Je     GGTTGCAATTTCTGGTTACCAAAAGCTCATGTAGAAGCAAGTACTAGAGGTTCTATAAAT
*****

Xi-Fr-SE1      TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Fr-Mel1    TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Ir-Ta-A    TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Ir-Ta-B    TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Ir-Sh      TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Eg-Is     TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Tu-Al-B   TTGGCAGGCTACTTTTTAAAGTTGGGGGGGTTTGGGGCTATTCGATTAAAAAGTTTATT
Xi-Gr-Sa-AB  TTGGCTGGTCTACTTTTTAAAGTTGGGGGGGTTTGGAGCTATTCGATTAAAAAGTTTATT
Xi-It-PC     TTGGCTGGTCTACTTTTTAAAGTTGGGGGGGTTTGGAGCTATTCGATTAAAAAGTTTATT
Xi-CJ-Je     TTGGCTGGTCTACTTTTTAAAGTTGGGGGGGTTTGGAGCTATTCGATTAAAAAGTTTATT
*****

Xi-Fr-SE1      TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Fr-Mel1    TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Ir-Ta-A    TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Ir-Ta-B    TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Ir-Sh      TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Eg-Is     TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Tu-Al-B   TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-Gr-Sa-AB  TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-It-PC     TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
Xi-CJ-Je     TTTGTAAGGGATTAGTTTGGTTCCTGTTTTTTACTAGCTTTAGTCGCTAGTTAGTC
*****

Xi-Fr-SE1      ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Fr-Mel1    ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Ir-Ta-A    ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Ir-Ta-B    ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Ir-Sh      ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Eg-Is     ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Tu-Al-B   ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-Gr-Sa-AB  ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-It-PC     ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
Xi-CJ-Je     ACTTGTGTGCTGATCTGATACTAAGAAGCTAGTAGCCTATAGCAGTGTGACTCATATAACA
*****

Xi-Fr-SE1      TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Fr-Mel1    TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Ir-Ta-A    TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Ir-Ta-B    TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Ir-Sh      TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Eg-Is     TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Tu-Al-B   TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGAGTAATCATTTTTA
Xi-Gr-Sa-AB  TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGGTAATCGTTTTTA
Xi-It-PC     TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGGTAATCGTTTTTA
Xi-CJ-Je     TTTATGGTTTTGGGTTTGGTTAGGAGGCTAAAGAAAGGCTTAATTAGGTAATCGTTTTTA
*****

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Xi-Fr-SE1 TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Fr-Me1 TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Ir-Ta-A TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Ir-Ta-B TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Ir-Sh TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Eg-Is TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Tu-Al-B TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-Gr-Sa-AB TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-It-PC TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT
 Xi-CJ-Je TCCTTAGCTCATGGTTGAGCTTCTTCAGGCATATTTTAGCTATGGGTGCTATAAGGGGT

Xi-Fr-SE1 ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Fr-Me1 ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Ir-Ta-A ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Ir-Ta-B ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Ir-Sh ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Eg-Is ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Tu-Al-B ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-Gr-Sa-AB ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-It-PC ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA
 Xi-CJ-Je ATTTCGAAGTCTCGTCTTCTTTTCTTGTGAGATCAGAAAGAAAGTTTACTTTTTTTTA

Xi-Fr-SE1 TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Fr-Me1 TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Ir-Ta-A TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Ir-Ta-B TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Ir-Sh TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Eg-Is TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Tu-Al-B TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-Gr-Sa-AB TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-It-PC TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC
 Xi-CJ-Je TTGATTTTGGGTTAATTTTAGTTTTAAATTCCTCTATTCCTCC

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