

Great molecular variation within the species *Phytoseius finitimus* (Acari: Phytoseiidae): implications for diagnosis decision within the mite family Phytoseiidae

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ABSTRACT — Molecular markers are increasingly used for species identification and new taxa description. However, rules to determine frontiers between populations and species are not clear depending on taxa considered. For mites, few studies deal with molecular diagnoses, making associated decision' rules difficult. The present study focuses on a species of the predatory mite family Phytoseiidae (*Phytoseius finitimus*), considered for biological control of mites and small insect pests in fruit orchards and vineyards in the Mediterranean basin. This paper aims to elucidate the causes of great molecular variations and questions the occurrence of cryptic species. Molecular (12S rRNA, CytB mtDNA, ITSS) and morphological analyses were performed on four populations collected in Corsica and Italy in crops (vine and kiwi) and in an uncultivated environment (*Viburnum lantana*). Different methods for identifying species have been used (tree approaches, distances and ABGD algorithms). A reference database of distances within and between Phytoseiidae species has been elaborated to inform the present question and to assist with further diagnosis within Acari. Mitochondrial DNA analyses show that specimens from *V. lantana* were well separated from the three other populations with high genetic distances, suggesting the existence of a cryptic species. Molecular ITSS analyses coupled with morphological features show however that the four populations seem to belong to the same species. The great mitochondrial polymorphism is discussed in regards to: (i) genetic distances reported for Phytoseiidae species and (ii) potential biological differences between populations (cultivated *versus* uncultivated areas). This study clearly emphasizes the necessity of integrative taxonomy approaches for diagnosis decisions. Furthermore, based on the polymorphism herein detected, maximal intraspecific distances are proposed (9, 23 and 2.8 % for 12S rRNA, CytB mtDNA and ITSS) for diagnosis decisions within Phytoseiidae. Further statistical analyses are however clearly required to determine statistical error for general and reliable decision making.

KEYWORDS — Acari; molecular taxonomy; morphometrics; species delineation; integrative taxonomy

INTRODUCTION

Molecular markers for differentiating microorganism, plant and animal species are currently used in taxonomy. The rapid technical developments (*i.e.* DNA extraction, PCR and Sanger sequencing) during these last decades as well as barcoding approaches and associated conceptual definitions have led to the development of molecular diagnosis (*i.e.* Hebert *et al.* 2003a,b; 2004a,b; DeSalle *et al.* 2005; Goldstein and DeSalle 2011). Molecular markers were traditionally used when doubts on species identity arise from morphological comparisons and sometimes from biological features between populations. However, cryptic species are more and more revealed *via* comparison of DNA sequences of apparently morphologically similar specimens (*i.e.* Pons *et al.* 2006; Witt *et al.* 2006; Bickford *et al.* 2007; Valentini *et al.* 2009; Footitt *et al.* 2009; Pauls *et al.* 2010; Jorger *et al.* 2012; Kekkonen and Hebert, 2014; Kekkonen *et al.* 2015). The present study focuses on a species of the mite family Phytoseiidae: *Phytoseius finitimus* Ribaga. This family, containing 2,479 valid species (Demite *et al.* 2016), is one of the most well-known within the Mesostigmata, due to the predatory abilities of numerous species to control mite and small insect pests (McMurtry and Croft 1997; Gerson *et al.* 2003; McMurtry *et al.* 2013). Correct identification of these predators is clearly of huge importance for ensuring the success of biological control, as different species have different biological features (*i.e.* Tixier *et al.* 2006; McMurtry *et al.* 2013). Phytoseiidae species identification is essentially based on female morphological characters. However, the reliability of those characters as well as the low number of morphological features potentially available (due to the mite small size, 500 μm maximum in length) have led to the development of molecular studies to resolve questions concerning synonymies (*i.e.* Jeyaprakash and Hoy 2002; Noronha *et al.* 2003; Tixier *et al.* 2006, 2008a, 2010a, 2011b, 2012, 2014; Okassa *et al.* 2009, 2010, 2011, 2012; Kanouh *et al.* 2010a,b; Bowman and Hoy 2012; Chao *et al.* 2012; Navia *et al.* 2014; Rezende *et al.* 2015). For ensuring molecular diagnosis, a database containing DNA sequences associated to the correct species name is essential (*i.e.*

DeSalle *et al.* 2005; Collins and Cruickshank 2013). When elaborating such a database for Phytoseiidae species, high molecular variations were observed within specimens identified as *P. finitimus* during samplings carried out in Italy and France, two areas where this species is most commonly found.

Phytoseius finitimus is a generalist predator and is quite common in Mediterranean vineyards (*i.e.* Ragusa and Ciulla 1991; Duso and Vettorazzo 1999; Duso and Fontana 2002; Peverieri *et al.* 2009; Miñarro and Kreiter 2012). Some studies have shown its ability to control phytophagous mites, whiteflies and thrips and to develop when fed with pollen (Pappas *et al.* 2013) and Ahmad *et al.* (2015) recently studied its interspecific competition features. The present study aims to elucidate the great molecular distances observed between four *P. finitimus* populations and to determine how these great variations are related or not to cryptic species occurrence. Answering this question is clearly important for biological control applications, as implementations and advice for agro-environmental management would be different if one or two species are named under *P. finitimus*. To determine if one or several species were present, an integrative taxonomic approach based on three molecular fragments and morphological analyses has been carried out. The number of analytical developments for assessing species within a DNA sequence dataset is increasing for ensuring diagnosis decision (*i.e.* Kekkonen *et al.* 2015). Traditional cluster tree-based and distance threshold-based approach as well as the overlapping ABGD (Automatic Barcode Gap Discovery) algorithm have been herein used to determine their respective performance and congruence. Furthermore, a compilation of genetic distances between Phytoseiidae species retrieved from already published works is also provided as a reference comparison point. This review aims to provide additional information that can be used in informing the decision and more broadly aims to assist further analyses on DNA molecular diagnosis within the family Phytoseiidae for delimiting intra- and interspecific frontiers.

MATERIALS AND METHODS

Species and populations studied

Phytoseius finitimus, even if reported many times in the literature, is not naturally observed in great densities and is quite difficult to efficiently retrieve. It is known to be frequent in Italy and in Corsica (France), especially in vineyards (Kreiter *et al.* 2000; Duso and Fontana 2002; Demite *et al.* 2014). Four populations of *P. finitimus* were considered, with the double aims to study populations (i) from the two main areas where this species is most abundant (Italy and France) and (ii) from cultivated (vine and kiwi) and uncultivated areas (colonized by *Viburnum lantana* L.). Two populations were collected in Italy in the same locality on vines (*Vitis vinifera* L.) and on uncultivated *V. lantana* plants located besides the vine plot. Two populations were collected in France – Corsica, on vine and kiwi (*Actinidia deliciosa* C.F. Liang & A.R. Ferguson.) on plots separated by approximately 15 kms (Figure 1). The characteristics of the populations studied and accession numbers of DNA sequences in the Genbank database are presented in Table 1. The species *Typhlodromus* (*Typhlodromus*) *phialatus* Athias-Henriot, belonging to the sub-family Typhlodrominae, was used as an out-group for tree construction. Sequences of this latter species were obtained by present authors and already referenced in the Genbank database (12S rRNA: HM635274, ITSS: HQ404829, CytB mtDNA: HM635300) (Okassa *et al.* 2012; Tsolakis *et al.*, 2012).

Molecular experiments

DNA extraction.

Total genomic DNA of a single female was extracted using a Qiagen DNeasy tissue kit (Qiagen, Hilden, Germany), according to the DNA extraction protocol « Purification of Total DNA from Animal Blood or Cells » (Spin-Column Protocol) adapted for extracting total DNA from mites (Kanouh *et al.* 2010b). Between six and thirteen females were considered per population (Table 1). After extraction, specimens were retrieved and mounted on slides according the method developed by Tixier *et al.* (2010 b).

DNA amplification and sequencing.

A nuclear ribosomal gene section including ITS1-5.8S-ITS2 (reported as ITSS) and two mitochondrial markers (12S rRNA, Cytochrome B mtDNA) were amplified within a multi-barcoding approach to avoid the current pitfall of barcoding using only one small DNA fragment (Moritz and Cicero 2004; Collins and Cruickshank 2013). These DNA fragments have been used for species diagnosis within Phytoseiidae (Jeyaprakash and Hoy 2002; Tixier *et al.* 2006, 2008a, 2010a, 2011b, 2012, 2014; Okassa *et al.* 2009, 2010, 2011, 2012; Kanouh *et al.* 2010a,b; Navia *et al.* 2014; Rezende *et al.* 2015). Primers and thermal cycling are those reported in Tixier *et al.* (2012). The PCR reactions were performed in a 25 µL volume, containing 4 µL of mite DNA for mt markers and 2 µL for ITSS, 2.5 µL (1 mM) of buffer 10X, 1 µL (1.5 mM) of MgCl₂, 0.5 µL (0.05 mM for each) DNTPs, 0.175 µL (0.7 µM) for each primer, 0.125 µL (0.625 U) of Taq Qiagen and 16.525 µL of water. Electrophoresis was carried out on a 1.5 % agarose gel in 0.5 X TBE buffer during 20 min at 135 volts. PCR products were purified using ExoSAP-IT (Amersham) and sequenced along both strands using Dynamic ET Terminator Cycle Sequencing kit. The sequencer used was the Megabase 1,000 apparatus. Sequences were aligned and analysed with Geneious v3.5.4 (Drummond *et al.* 2007).

Data analyses.

A preliminary analysis was conducted on the coding sequences (CytB mtDNA) to check for the absence of stop codons. Three molecular diagnosis approaches were used to assess the occurrence of cryptic species within the four populations of *P. finitimus*.

Tree-based cluster approach.

Neighbour-joining trees were constructed using the Kimura 2-parameter model and node support was determined using 1,000 bootstrap replicates calculated in Mega 6.0.6® (Tamura *et al.* 2013). The K2P model was used to make our results comparable with other studies on Phytoseiidae. Furthermore to determine how other tree models perform for resolving the question on cryptic species, Parsimony and Bayesian analyses were carried out for

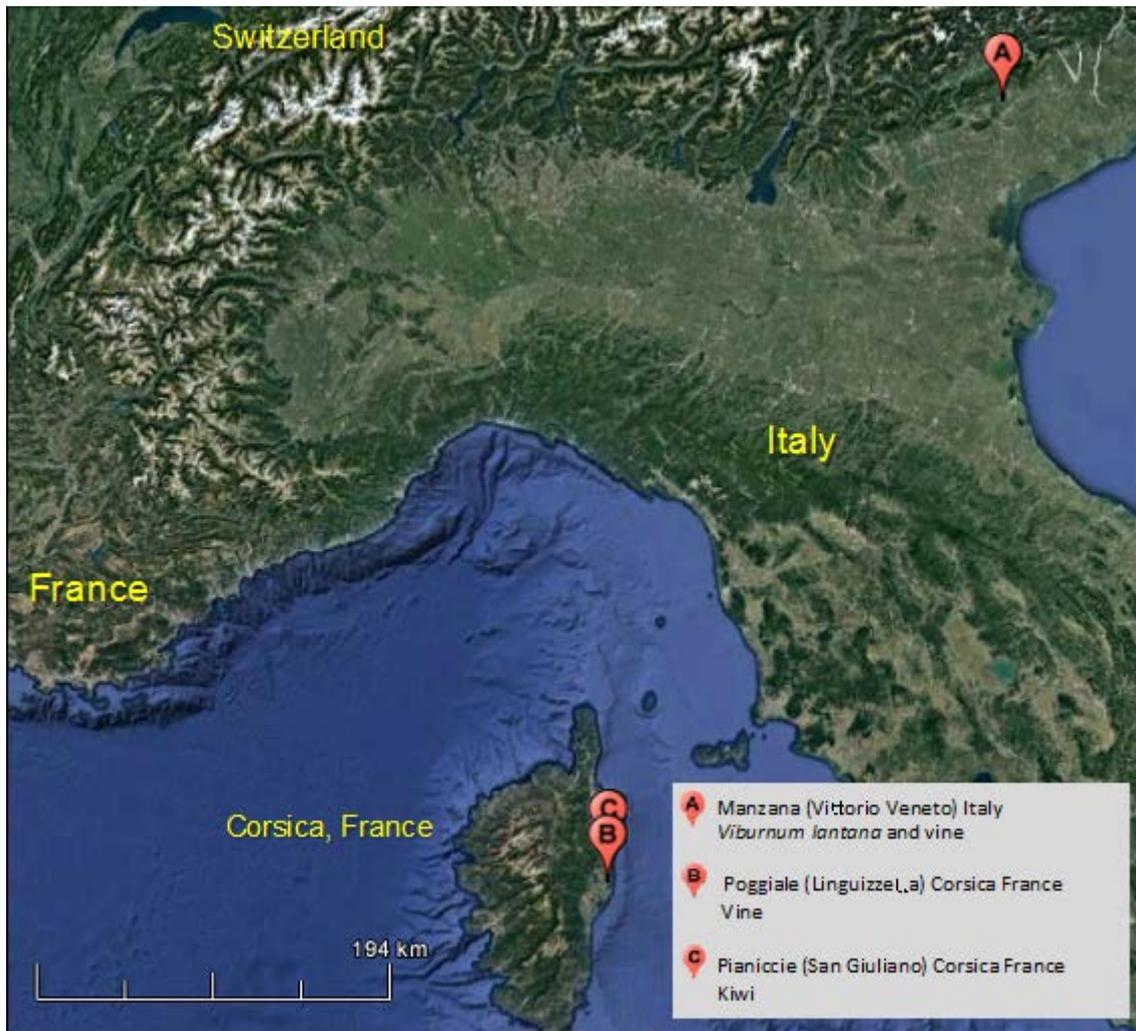


FIGURE 1: Map showing the localities where the four populations of *Phytoseius finitimus* have been collected (on kiwi and vine in Corsica, on *Viburnum lantana* and vine in Italy).

each fragment and combined dataset (applying simple partition procedure with one partition corresponding to each DNA marker). As for some specimens, it was impossible to obtain PCR products (especially for ITS), the combined data set was analysed following Wiens (1998a,b), Wiens *et al.* (2005) and Zheng and Wiens (2016), who demonstrated that missing data do not affect the phylogenetic accuracy.

The Incongruence Length Difference (ILD) P value ($P=0.07$) suggests no conflicting signals between markers (Mickey and Farris 1981; Sullivan 1996; Farris *et al.* 1994; Cunningham 1997). For

Parsimony analyses a heuristic search procedure repeated 1,000 times, was performed with randomized taxa additions and branch-swapping algorithm (TBR). To reduce misleading effects of homoplasious characters, an *a posteriori* re-weighting was applied according to the rescaled consistency index (RC) after each tree search, until the number of trees stabilised (Farris 1969, 1989) (PAUP*, v.4.0b.10 / Swofford, 2002). Bayesian analysis was carried out using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003); the best-fit-substitution models were determined by Modeltest 3.07 in PAUP (Posada and Crandall 1998) through hierarchical likelihood-ratio

TABLE 1: Characteristics of collection localities of the different populations, plant support species considered and accession numbers in the Genbank database.

Country	Locality	GPS coordinates	Host plant	Genbank accession numbers		
				12S rRNA	CytB mtDNA	ITSS
Italy	Manzana (Vittorio Veneto)	45°55'52.16"N 12°16'11.63"E, 168 m alt.	<i>Viburnum lantana</i> L.	KX021177	KX021132	KX021162
				KX021178	KX021133	KX021163
				KX021179	KX021134	KX021164
				KX021180	KX021135	KX021165
				KX021181	KX021136	KX021166
				KX021182	KX021137	KX021167
				KX021183	KX021138	KX021168
				KX021184	KX021139	
				KX021185		
			KX021190	KX021146	KX021170	
			KX021191	KX021147	KX021171	
			KX021192	KX021148	KX021172	
			KX021193	KX021149		
			KX021194	KX021150		
			KX021195	KX021151		
			<i>Vitis vinifera</i> L.	KX021196	KX021152	
				KX021197	KX021153	
				KX021198	KX021154	
				KX021199	KX021155	
KX021200	KX021156					
KX021201	KX021157					
KX021202	KX021158					
France	Pianiccie San Giuliano Corsica	42°16'53"N, 9°32'2"E, 25 m alt.	<i>Kiwi: Actinidia deliciosa</i> C.F. Liang & A.R. Ferguson	KX021173	KX021128	KX021159
				KX021174	KX021129	KX021160
				KX021175	KX021130	KX021161
				KX021176	KX021131	
France	Poggiale Linguizzetta Corsica	42°11'6"N, 9°29'59"E, 51 m alt.	<i>Vitis vinifera</i> L.	KX021186	KX021140	KX021169
				KX021187	KX021141	
				KX021188	KX021142	
				KX021189	KX021143	
					KX021144	
	KX021145					

tests (12S rRNA: HKY+G; CytB mtDNA: TIM+I; ITSS: HKY+G). The number of categories used to approximate the gamma distribution was set at four, and four Markov chains were run for 100,000 generations. Stabilisation of model parameters (burn-in) occurred around 250 generations.

As tree clades do not necessary reflect species clusters but can represent populations of a same species (i.e. Moritz and Cicero 2004; Collins and Cruickshank 2013), the classical threshold-based approach was applied and distance matrices (using the Kimura 2-parameter) model were elaborated. With this approach, ideally no overlap should occur

between intra and interspecific distances (i.e. Meyer and Paulay 2005). Even if the no-overlap model is sometimes point of contention especially because of sampling completion, knowledge of species considered and associated taxonomical features, distance comparison is currently used to distinguish between species for practical assignments. Two approaches for comparing distances were applied.

The overlap distance criteria.

First, distances within and between the four populations were manually compared to determine gap occurrence. In addition, the ABGD (Automatic Barcode Gap Discovery) algorithm

was applied. It aims at automatically determining the occurrence of different species within a dataset based on gap between batches of sequences pairs (Puillandre *et al.* 2012a), detecting the first barcode gap and using it to partition the dataset into candidate species. Analyses were performed in November 2016 on the web interface (<http://wwwabi.snv.jussieu.fr/public/abgd/>). They were conducted using three metrics (Jukes-Cantor (JC), Kimura 2 parameter (K2P) and simple p-distances) and default parameter values.

Empirical comparisons to references.

Distances usually constitute a useful point of comparison when the taxonomic status of the taxa compared is sure (Robinson *et al.* 2009). Thus, the genetic distances herein obtained were compared to those mentioned in 15 publications for 24 Phytoseiidae species. Only distances issued from published works and not calculated from all Phytoseiidae sequences deposited in Genbank were considered, because of great incertitude on the true identity of the sequences deposited in this world database (i.e. Tixier *et al.* 2011a). Two distances were compiled: (i) one corresponding to the maximal value observed within a same species (maximal intraspecific variation) and (ii) one corresponding the minimal value observed between two species of a same genus [minimal interspecific variation assumed to be the "Nearest Neighbour" (Kekkonen *et al.* 2015)].

Morphological analyses

Characters considered.

As female dorsal seta lengths are currently used to discriminate between Phytoseiidae species (Chant and McMurtry 2007), the seventeen dorsal idiosomal setae were measured for 5 to 10 females issued from the same populations as sequenced specimens: *j1*, *j3*, *j4*, *j5*, *j6*, *J2*, *J5*, *z2*, *z3*, *z5*, *z4*, *Z4*, *Z5*, *s4*, *s6*, *r3* and *R1*. The lengths of the seta *JV5* and the macroseta on the basitarsus of the leg IV (*St IV*) were also considered. Finally, peritreme, spermatheca, ventrianal shield and chelicera shapes were observed. Terminology for seta notation follows that of Lindquist and Evans (1965) as adapted by Rowell *et al.* (1978) for Phytoseiidae.

Data analyses. The data corresponding to the 17 continuous characters were normally distributed and variances were equal (Bartlett test). ANOVA analyses were performed for each character to determine differences between the four populations. A principal component analysis was also carried out to determine whether the combination of the morphological characters differentiates specimens considered. All statistical analyses were carried out with Statistica (Statsoft France 2010).

To determine the variation of each seta length, the rCL_{95} corresponding to the percentage of variation around the mean was calculated according to the formula provided in Tixier (2012). Furthermore, min and max values of the interval assumed to include 95 % of specimens of a same species for each setal length were modelled using the abacus provided in Tixier (2012) to determine whether the observed variation would be included in the modelled "normal" intraspecific variation.

RESULTS

Molecular analyses.

437, 418 and 690 base pairs (bp) were aligned for the 12S rRNA, Cytb mtDNA, and ITSS genes, respectively. Quite similar and constant rates of nucleotide substitutions were observed for all the populations studied. A BLAST search in Genbank showed that the sequences aligned with those of Phytoseiidae. The overall genetic distances between the specimens of *P. finitimus* for 12S rRNA (mean = 2.9 %, min-max = 0-9 %) and CytB mtDNA (mean = 7.6 %, min-max = 0-23 %) are high in regards to those obtained in other studies (Table 3) questioning the occurrence of cryptic species.

Diagnosis decision and tree cluster criteria.

The same tree topologies were observed with the three algorithms (Neighbour Joining, Parsimony, Bayesian analyses) for the three markers considered. Three clades were observed on the trees obtained with the two mitochondrial markers (12S rRNA, CytB mtDNA): one containing specimens collected in Corsica on vine, one containing specimens collected in Italy on vine and in Corsica on kiwi and

TABLE 2: Mean genetic K2P distances (minimal and maximal values), between and within the four populations considered (kiwi-Corsica, *Viburnum lantana*-Italy, vine-Italy and vine-Corsica) for the three molecular markers used 12S rRNA (a), CytB mtDNA (b) and ITSS (c).

(a)

	Vine Italy	Kiwi Corsica	Vine Corsica	<i>Viburnum lantana</i> Italy
vine Italy	0 (0-0.017)			
Kiwi Corsica	0.002 (0-0.02)	0.02 (0-0.02)		
Vine Corsica	0.04 (0.03-0.05)	0.04 (0.03-0.04)	0.02 (0-0.03)	
<i>Viburnum lantana</i> Italy	0.05 (0.04-0.06)	0.05 (0.05-0.06)	0.07 (0.06-0.090)	0.003 (0-0.008)

(b)

	Vine Italy	Kiwi Corsica	Vine Corsica	<i>Viburnum lantana</i> Italy
vine Italy	0.006 (0-0.022)			
Kiwi Corsica	0.006 (0-0.018)	0.004 (0.003-0.005)		
Vine Corsica	0.08 (0.003-0.16)	0.08 (0.003-0.14)	0.06 (0.003-0.13)	
<i>Viburnum lantana</i> Italy	0.12 (0.11-0.16)	0.12 (0.10-0.16)	0.16 (0.10-0.23)	0.023 (0-0.06)

(c)

	vine Italy	Kiwi Corsica	Vine Corsica	<i>Viburnum lantana</i> Italy
vine Italy	0.002 (0-0.003)			
Kiwi Corsica	0 (0-0)	0 (0-0)		
Vine Corsica	0 (0-0)	0 (0-0)	-	
<i>Viburnum lantana</i> Italy	0.008 (0.003-0.028)	0.006 (0.003-0.028)	0.005 (0.003-0.017)	0.007 (0-0.025)

TABLE 3: Results of the ABGD analyses with the groups obtained and Prior maximal distance and number of partitions.

	12S rRNA	CytB mtDNA	ITSS
Jukes-Cantor (JC69) and Kimura (K80) TS/TV distances	4 groups: 9 sequences: <i>V. lantana</i> 17 sequences: Vine Italy and kiwi 2 sequences: Vine Corsica 2 sequences: Vine Corsica	5 groups: 8 sequences: <i>V. lantana</i> 17 sequences: Vine Italy and kiwi 4 sequences: Vine Corsica 1 sequence: Vine Corsica 1 sequence: Vine Corsica	1 group
Prior maximal distance	7 partitions, $P = 0.02$	8 partitions, $P = 0.03$	2 partitions, $P = 0.001$
P distance	2 groups: 9 sequences: <i>V. lantana</i> 21 sequences: other populations	2 groups: 8 sequences: <i>V. lantana</i> 23 sequences: other populations	1 group
Prior maximal distance	5 partitions, $P = 0.007$	7 partitions, $P = 0.02$	1 partition, $P = 0.001$

one containing specimens collected in Italy on *V. lantana* (Figures 2, 3). One specimen collected on vine in Corsica was however located in the clade containing specimens from vine-Italy and kiwi-Corsica. These clades could be three species or three populations of a same species. Specimens included in the two former clades are more closer to each other than to specimens included in the third. Two not

well-sustained clades are observed on the ITSS tree (Figure 4), one containing specimens from *V. lantana* and one containing the specimens of the three other populations. Furthermore, the same clades as those obtained with mitochondrial markers were also obtained when concatenated data were considered for both Parsimony and Bayesian analyses (Figure 5).

Diagnosis decision and the overlap criteria.

Figure 6 presents the four intra-populations distances and the six inter-population distances. For 12S rRNA, overlap is observed between intra-population distances and distances between specimens collected on vine in Italy, vine and kiwi in Corsica. However, no overlap is observed between intra-population distances and distances between specimens collected on *V. lantana* in Italy and those from the three other populations (Figure 6). For CytB mtDNA, overlap is observed between intra-population and all inter-population distances, especially because of the high intra-population distances observed within the vine-France population. Finally, for ITSS marker, overlap is observed between all intra and inter-population distances.

With the ABGD analyses, the same groups were obtained for the Jukes-Cantor (JC69) and Kimura (K80) TS/TV distances but different clusters were observed for the P distance (Table 3). For the 12S rRNA and CytB mtDNA markers, four and five groups were observed with the K80 and JC69 distances, respectively. Globally, the same groups as those observed on the phylogenetic trees were obtained, but more clustering was observed within the population collected on vine in Corsica (two clusters for 12S rRNA and three clusters for CytB mtDNA). Using P distance, only two groups were observed with the two mitochondrial markers, one containing specimens collected on *V. lantana* and one containing all the others. For the ITSS marker, one group was observed whatever the distance considered. The ABGD method provides thus different information and groups depending on (i) mitochondrial DNA vs nuclear markers and (ii) the distance considered.

Diagnosis decision and comparison to references in literature.

The 12S molecular distances between clades I and II (0-5 %) correspond to values currently retrieved in literature for specimens belonging to the same species (Table 4). Furthermore, such distances are lower than the minimal distances observed until now between species of a same genus. In one case corresponding to distances between *Phytoseiulus persimilis* Athias-Henriot and *P. macropilis* (Banks),

the presently observed distances are higher than the minimal distance observed between these two species (4 %). The maximal 12S rRNA distances between *V. lantana* specimens and specimens of clades I and II (9 %) is higher than the maximal intraspecific distances observed until now within a Phytoseiidae species (8 %) (Tables 2, 4). This value even if close to minimal distances observed between *Neoseiulus californicus* and *N. fallacis* (Garman) [10 % in Tixier *et al.* (2014) and 9.5 % in Jeyaprakash and Hoy (2002)] is however lower than distances observed until now between two Phytoseiidae species of the same genus.

The mean CytB distances between specimens from clades I and II are usually lower than genetic distances observed within a same Phytoseiidae species (Tables 2, 4). However, maximal distances (16 %) observed between specimens from vine in Italy and France, and kiwi from France as well those within vine-Corsica population (13 %), are higher or equal to the maximal intraspecific distances observed until now for Phytoseiidae (13 % for *N. aceri*). The maximal CytB distances between specimens from *V. lantana* and the three other populations (23%) are higher than those already observed within a same Phytoseiidae species. This value is just below the minimal interspecific distances observed between species of some genus (23.4 % between *T. (T.) phialatus* and *T. (T.) exhilaratus* and 24 % between *N. californicus* and *N. picanus* in Okassa *et al.* (2011, 2012)).

The highest ITSS distances are observed between specimens collected on *V. lantana* and the specimens of the other populations. The distances between clades I and II are very low and included in the variation range of intraspecific distances retrieved in literature (Table 3). The mean distances between these two clades and specimens from *V. lantana* are usually lower than those observed between different species of a same Phytoseiidae genus. The maximal distance is however similar to the distances obtained between *T. (T.) phialatus* and *T. (T.) exhilaratus* (two morphological close species) by Tixier *et al.* (2006) whereas Navia *et al.* (2014) reported a distance of 4.75 % between these two same latter species (Table 4).

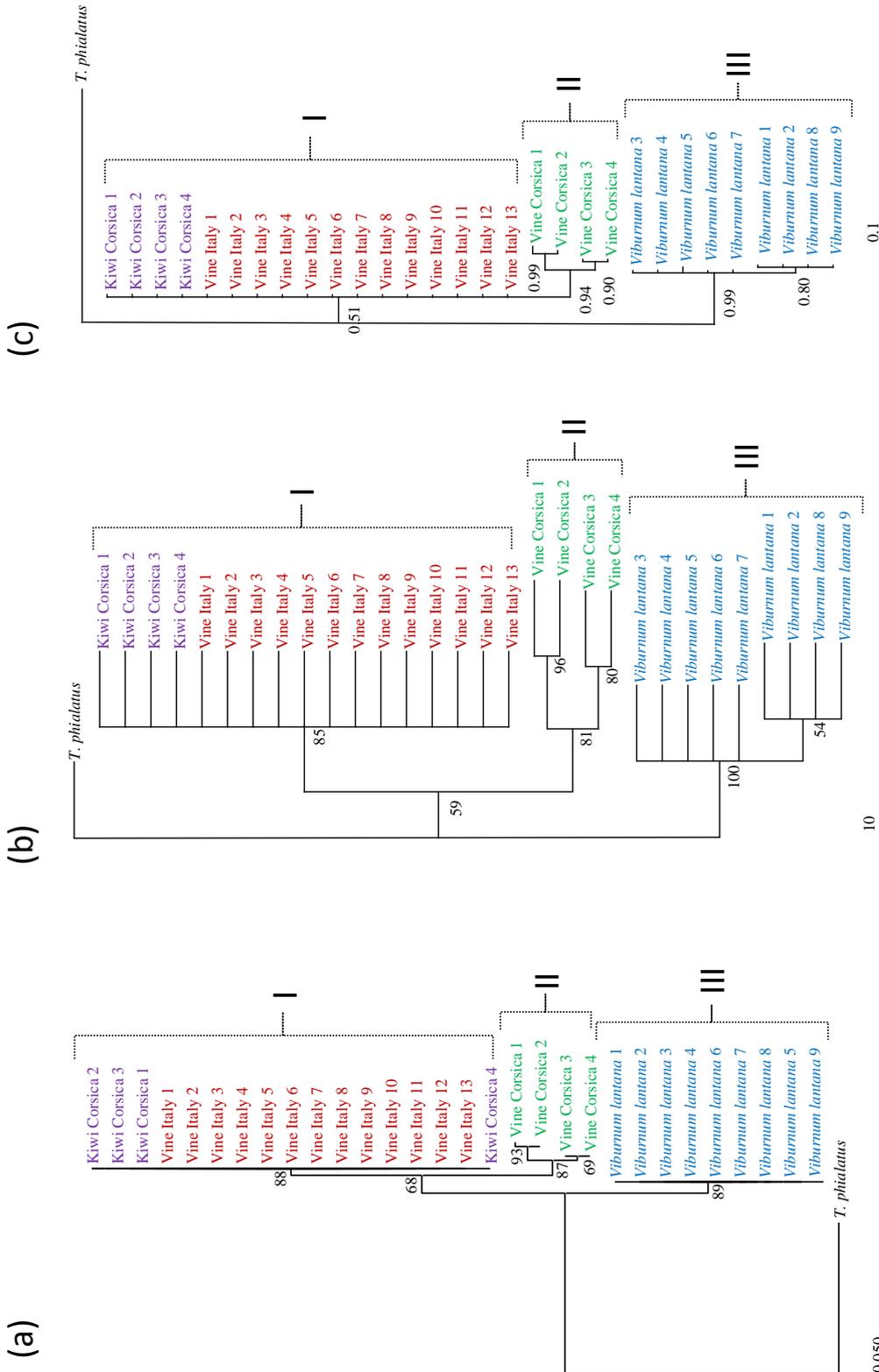


FIGURE 2: Phylogenetic trees obtained with (a) Neighbour Joining, (b) Parsimony (Consistency Index = 0.97) and (c) Bayesian analyses using the 12S rRNA fragment for specimens collected on kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica. The numbers at nodes correspond to bootstrap values (Neighbour Joining, Parsimony) and posterior probability (Bayesian).

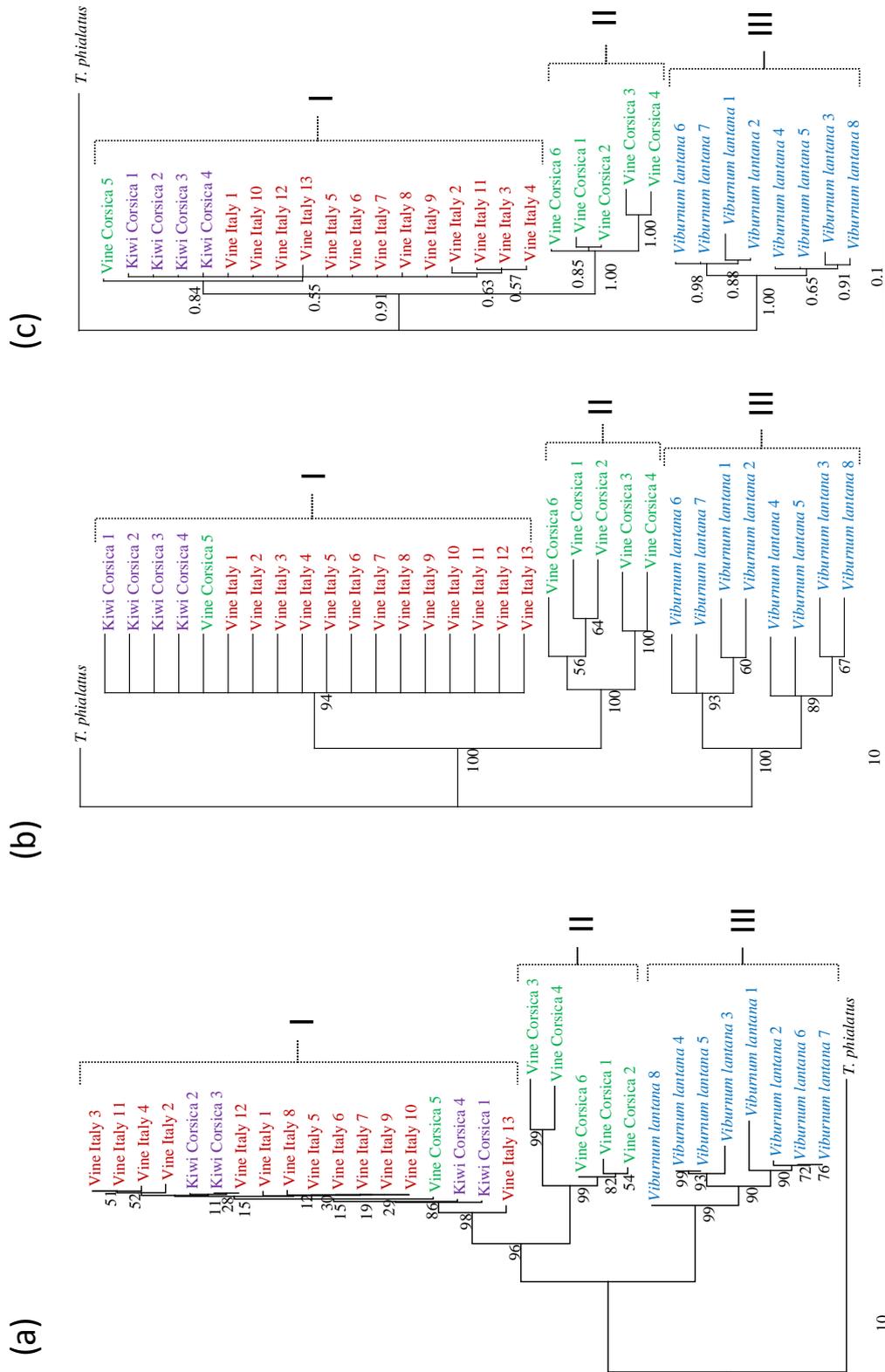


FIGURE 3: Phylogenetic trees obtained with (a) Neighbour Joining, (b) Parsimony (Consistency Index = 0.92) and (c) Bayesian analyses using the Cytb mtDNA fragment for specimens collected on kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica. The numbers at nodes correspond to bootstrap values (Neighbour Joining, Parsimony) and posterior probability (Bayesian).

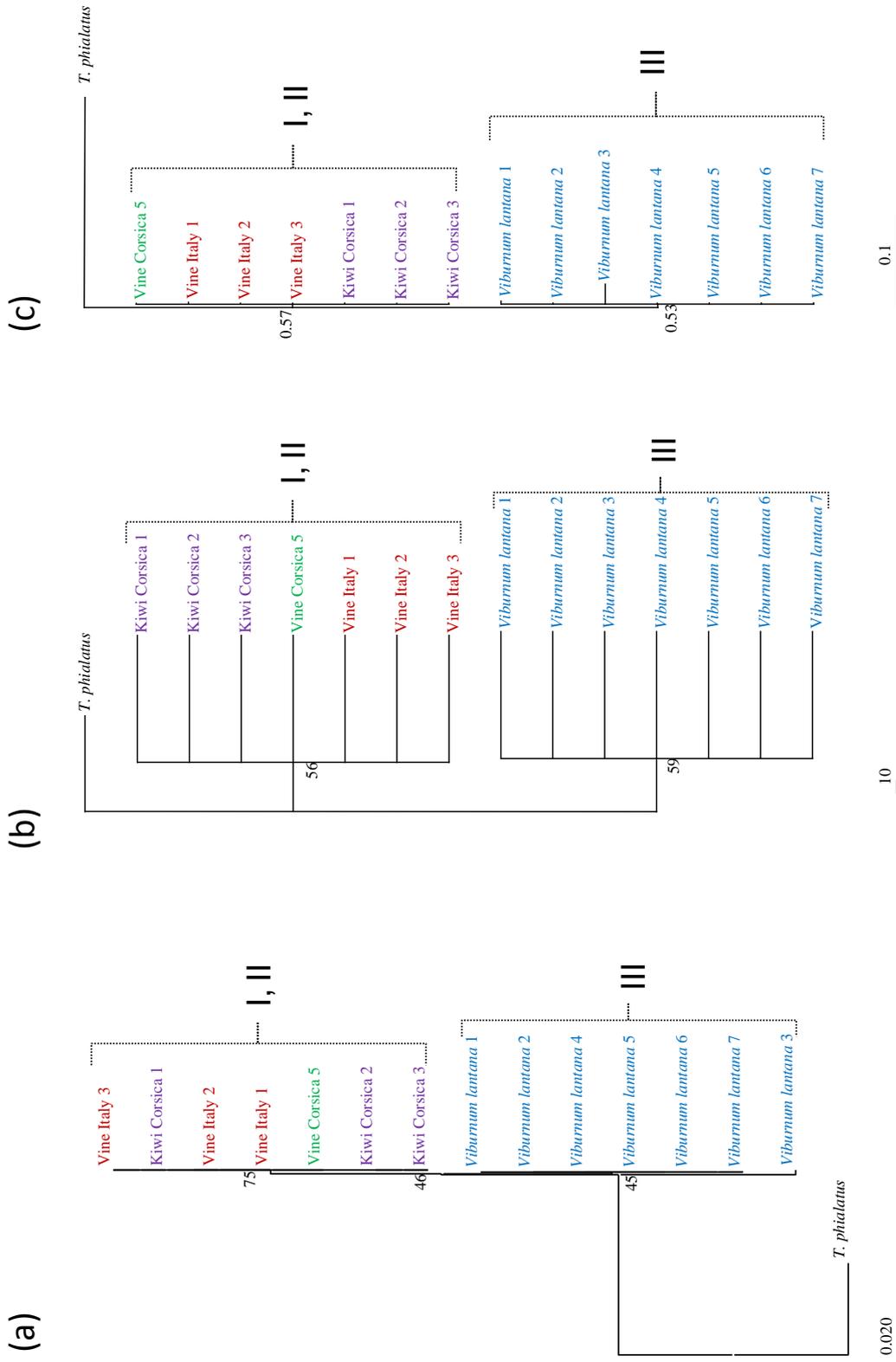


FIGURE 4: Phylogenetic trees obtained with (a) Neighbour Joining, (b) Parsimony (Consistency Index = 1.00) and (c) Bayesian analyses using the ITSS fragment for specimens collected on kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica. The numbers at nodes correspond to bootstrap values (Neighbour Joining, Parsimony) and posterior probability (Bayesian).

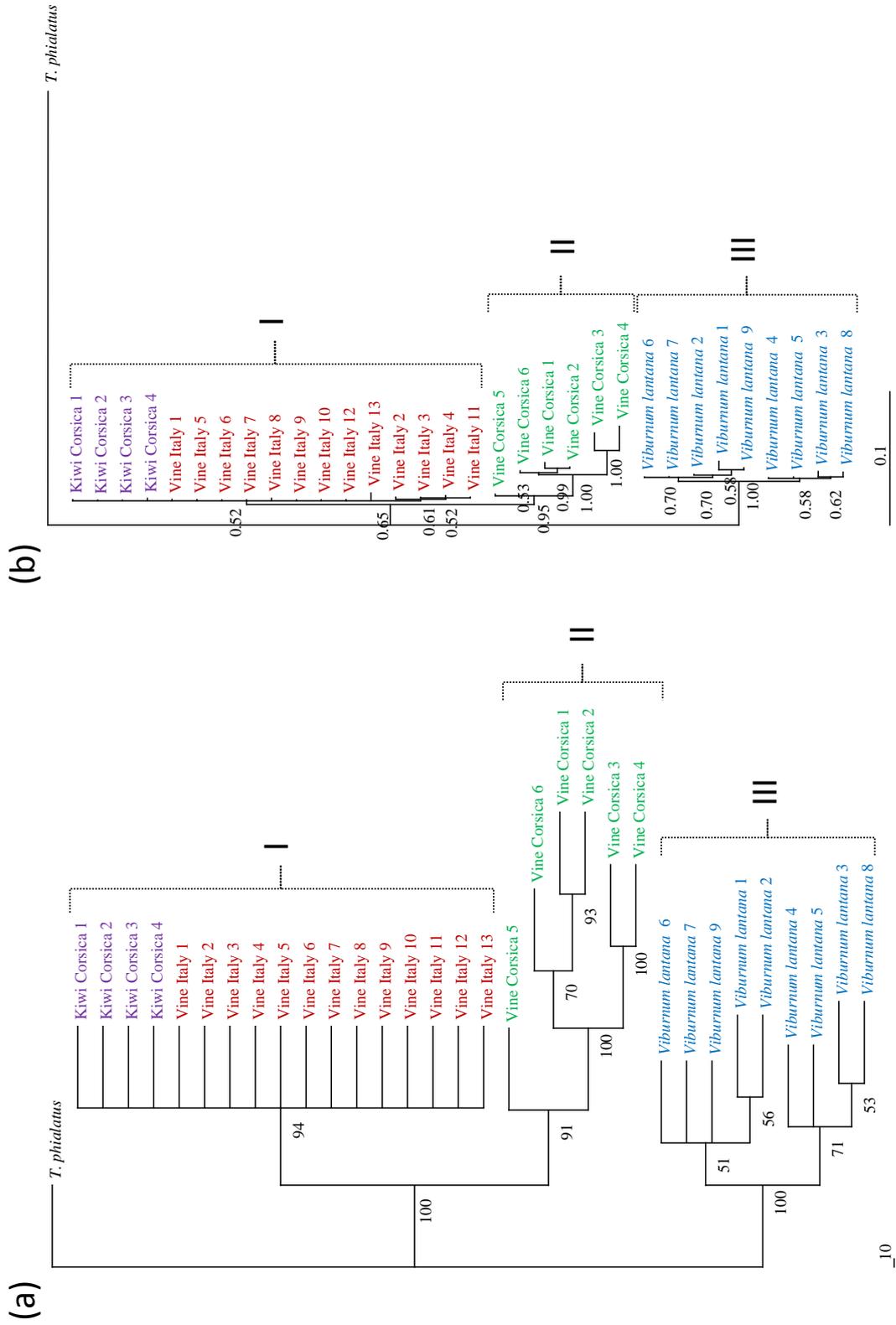


FIGURE 5: Phylogenetic trees obtained with (a) Parsimony (Consistency Index = 0.96) and (b) Bayesian analyses using the concatenated data (12S rRNA, CytB mtDNA, ITSS fragments) for specimens collected on kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica. The numbers at nodes correspond to bootstrap values (Parsimony) and posterior probability (Bayesian).

TABLE 4: Maximal intraspecific distances within Phytoseiidae species and minimal interspecific distances between Phytoseiidae species of the same genus reported in literature for the DNA markers: 12S rRNA, CytB mtDNA and ITSS.

	Maximal intraspecific distances			References
	12S	CytB	ITSS	
<i>Typhlodromus (Typhlodromus) pyri</i>	4.7%	10%	1.2%	Tixier <i>et al.</i> 2012
<i>Typhlodromus (Typhlodromus) exhilaratus</i>	0.3%	0.3%		Okassa <i>et al.</i> 2012
<i>Typhlodromus (Typhlodromus) phialatus</i>	0%	0%		Okassa <i>et al.</i> 2012
<i>Euseius finlandicus</i>	3%			Okassa <i>et al.</i> 2009
<i>Euseius stipulatus</i>	0%			Okassa <i>et al.</i> 2009
<i>Euseius gallicus</i>	0%			Okassa <i>et al.</i> 2009
<i>Neoseiulella aceri</i>	8%	13%	1%	Kanouh <i>et al.</i> 2010b
<i>Neoseiulella litoralis</i>	2.6%	5%	1%	Kanouh <i>et al.</i> 2010b
<i>Neoseiulella tiliarum</i>	1%		0%	Kanouh <i>et al.</i> 2010b
<i>Neoseiulus californicus</i>	5.2%	10.5%	0.04%	Okassa <i>et al.</i> 2011
<i>Phytoseiulus persimilis</i>	0%	0%		Okassa <i>et al.</i> 2010
<i>Phytoseiulus macropilis</i>	0%	0%		Okassa <i>et al.</i> 2010
<i>Phytoseiulus longipes</i>	0.4%			Tixier <i>et al.</i> 2010a
<i>Neoseiulus idaeus</i>	1%	1%	1%	Tixier <i>et al.</i> 2011
<i>Neoseiulus californicus</i>	0%			Tixier <i>et al.</i> 2014
<i>Neoseiulus idaeus</i>	0%			Tixier <i>et al.</i> 2014
<i>Neoseiulus californicus</i>	0%		0%	Navia <i>et al.</i> 2014
<i>Neoseiulus picanus</i>	0,10%		0.05%	Navia <i>et al.</i> 2014
<i>Amblyseius largoensis</i>	3%		0.16%	Navia <i>et al.</i> 2014
<i>Amblyseius herbiocolus</i>	0%		0%	Navia <i>et al.</i> 2014
<i>Euseius citrifolius</i>			2%	Noronha <i>et al.</i> 2003
<i>Euseius concordis</i>			3%	Noronha <i>et al.</i> 2003
<i>Neoseiulus paspalivorus</i>			0%	Rezende <i>et al.</i> 2015
<i>Euseius nicholsi</i>			0.2%	Chao <i>et al.</i> 2012
<i>Neoseiulus californicus</i>	0%			Jeyaprakash & Hoy 2002
<i>Neoseiulus cucumeris</i>	0%			Jeyaprakash & Hoy 2002
<i>Neoseiulus fallacis</i>	0%			Jeyaprakash & Hoy 2002
<i>Iphiseius degenerans</i>	0%			Jeyaprakash & Hoy 2002
<i>Phytoseiulus persimilis</i>	0%			Jeyaprakash & Hoy 2002
	Minimal interspecific distances			References
	12S	CytB	ITSS	
<i>T.(T.) exhilaratus / T. (T.) phialatus</i>			2.8%	Tixier <i>et al.</i> 2006
<i>T.(T.) exhilaratus / T. (T.) pyri</i>			5.4%	Tixier <i>et al.</i> 2006
<i>T. (T.) phialatus / T. (T.) pyri</i>			6.7%	Tixier <i>et al.</i> 2006
<i>T.(T.) exhilaratus / T. (T.) phialatus</i>	13.9%	23.4%		Okassa <i>et al.</i> 2012
<i>Euseius gallicus / Euseius finlandicus</i>	16%			Okassa <i>et al.</i> 2009
<i>Euseius stipulatus / Euseius gallicus</i>	14%			Okassa <i>et al.</i> 2009
<i>Euseius stipulatus / Euseius finlandicus</i>	16%			Okassa <i>et al.</i> 2009
<i>Neoseiulella aceri / Neoseiulella tiliarum</i>	18%		5.9%	Kanouh <i>et al.</i> 2010b
<i>Neoseiulella aceri / Neoseiulella litoralis</i>	37%	47%	6%	Kanouh <i>et al.</i> 2010b
<i>Neoseiulella tiliarum / Neoseiulella litoralis</i>	41%		5.9%	Kanouh <i>et al.</i> 2010b
<i>Phytoseiulus macropilis / Phytoseiulus persimilis</i>	4%	15%		Okassa <i>et al.</i> 2010
<i>Phytoseiulus macropilis / Phytoseiulus fragariae</i>	13%	28%		Okassa <i>et al.</i> 2010
<i>Phytoseiulus fragariae / Phytoseiulus persimilis</i>	11%	24%		Okassa <i>et al.</i> 2010
<i>Phytoseiulus longipes / Phytoseiulus persimilis</i>	12%			Tixier <i>et al.</i> 2010
<i>Neoseiulus californicus / Neoseiulus idaeus</i>	16%	20%	4%	Tixier <i>et al.</i> 2014
<i>Neoseiulus californicus / Neoseiulus picanus</i>	12%	24%	4%	Okassa <i>et al.</i> 2011
<i>Neoseiulus idaeus / Neoseiulus californicus</i>	16%	20%	4%	Tixier <i>et al.</i> 2011
<i>Neoseiulus californicus / Neoseiulus idaeus</i>	12%			Tixier <i>et al.</i> 2014
<i>Neoseiulus californicus / Neoseiulus fallacis</i>	10%			Tixier <i>et al.</i> 2014
<i>Neoseiulus californicus / Neoseiulus picanus</i>	12%		4.17%	Navia <i>et al.</i> 2014
<i>Amblyseius largoensis / Amblyseius herbiocolus</i>	23%		5.89%	Navia <i>et al.</i> 2014
<i>T.(T.) exhilaratus / T. (T.) phialatus</i>	14%		4.75%	Navia <i>et al.</i> 2014
<i>Neoseiulus californicus / Neoseiulus picanus</i>	12%		4.17%	Navia <i>et al.</i> 2014
<i>Neoseiulus californicus / Neoseiulus fallacis</i>	9.5%			Jeyaprakash & Hoy 2002
<i>Phytoseiulus fintimus</i> max values	9%	23%	2.8%	

TABLE 5: Morphological measurements of the characters considered (μm) (mean, Standard deviation in parentheses, min-max values) for the four populations considered (kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica) and significance of between-population comparison. rCL₉₅ values (%) and min-max interval modelled according to Tixier (2012). sHIV: macroseta lengths on the bastitarsus of the leg IV.

	<i>Viburnum lantana</i> Italy (n=5)		<i>Vitis vinifera</i> Italy (n=5)		kiwi Corsica (n=10)		<i>Vitis vinifera</i> Corsica (n=10)		P value	Mean (min-max) for all populations	rCL ₉₅ (%)	min-max interval according to Tixier (2012)
	mean (SD)	min-max	mean (SD)	min-max	mean (SD)	min-max	mean (SD)	min-max				
<i>jl</i>	23 (3.35)	17-25	24 (1.37)	22-25	25 (1.42)	22-27	25 (0.79)	22-25	0.49	24 (17-27)	14	18-29
<i>j3</i>	49 (1.12)	47-50	51.5 (3.79)	47-57	55 (3.07)	50-57	56 (1.29)	55-57	0.00005	54 (47-57)	13	45-63
<i>j4</i>	10 (1.76)	7-12	9 (1.12)	7-10	10 (0.79)	10-12	10 (1.05)	10-12	0.44	10 (7-12)	22	7-13
<i>j5</i>	10 (0)	10	8 (1.37)	7-10	10 (0)	10	10 (0)	10	0.00008	10 (7-12)	15	7-13
<i>j6</i>	9 (1.12)	7-10	10 (1.77)	7-12	10 (0)	10	10 (0)	10	0.67	10 (7-12)	16	7-13
<i>J2</i>	11 (1.44)	10-12	12 (0)	12	13 (1.05)	13-15	13 (1.05)	13-15	0.025	13 (10-15)	19	9-16
<i>J5</i>	9 (1.44)	7-10	6 (1.44)	5-7	10 (0)	10	10 (0.79)	7-10	0.000001	9 (5-10)	33	6-12
<i>s4</i>	75 (5.30)	67-82	73 (6.22)	67-82	72 (3.98)	67-80	75 (1.67)	72-77	0.42	74 (67-82)	11	62-85
<i>s6</i>	81 (5.18)	75-87	88 (2.23)	87-92	89 (5.62)	75-95	94 (2.83)	90-100	0.00007	90 (75-100)	13	77-103
<i>z2</i>	10 (1.12)	10-12	10 (2.88)	7-12	14 (1.21)	12-15	12 (1.78)	10-15	0.00007	12 (7-15)	34	7-15
<i>z3</i>	30 (2.5)	27-32	28 (2.09)	25-30	33 (2.11)	30-37	34 (2.05)	32-37	0.00003	32 (25-37)	19	25-38
<i>z4</i>	12 (2.09)	10-15	13 (2.23)	10-15	17 (1.18)	15-20	15 (1.58)	12-17	0.00001	15 (10-20)	33	11-19
<i>z5</i>	9 (1.37)	7-10	10 (0)	10	12 (1.05)	10-12	10 (0.79)	10-12	0.00001	11 (7-10)	26	8-14
<i>Z4</i>	53 (2.74)	50-55	51 (4.78)	45-55	59 (3.29)	52-62	61 (5.65)	52-65	0.0015	58 (45-65)	19	48-68
<i>Z5</i>	84 (5.97)	75-90	86 (5.18)	80-92	83 (5.83)	70-87	84 (4.11)	77-87	0.63	84 (75-92)	12	71-96
<i>r3</i>	43 (7.15)	37-55	42 (4.67)	37-47	51 (1.29)	50-52	49 (1.05)	47-50	0.00005	48 (37-55)	21	38-56
<i>R1</i>	16 (3.35)	15-22	21 (2.39)	17-22	23 (1.69)	20-25	23 (1.21)	22-25	0.000007	22 (15-25)	29	17-27
<i>sHIV</i>	33 (3.25)	30-37	32 (5.77)	27-37	36 (2.41)	32-37	36 (2.12)	32-37	0.083	35 (27-37)	18	28-42
<i>JV5</i>	55 (6.22)	50-65	56 (6.98)	47-62	54 (1.21)	52-55	55 (0.79)	52-55	0.83	55 (47-65)	13	46-64

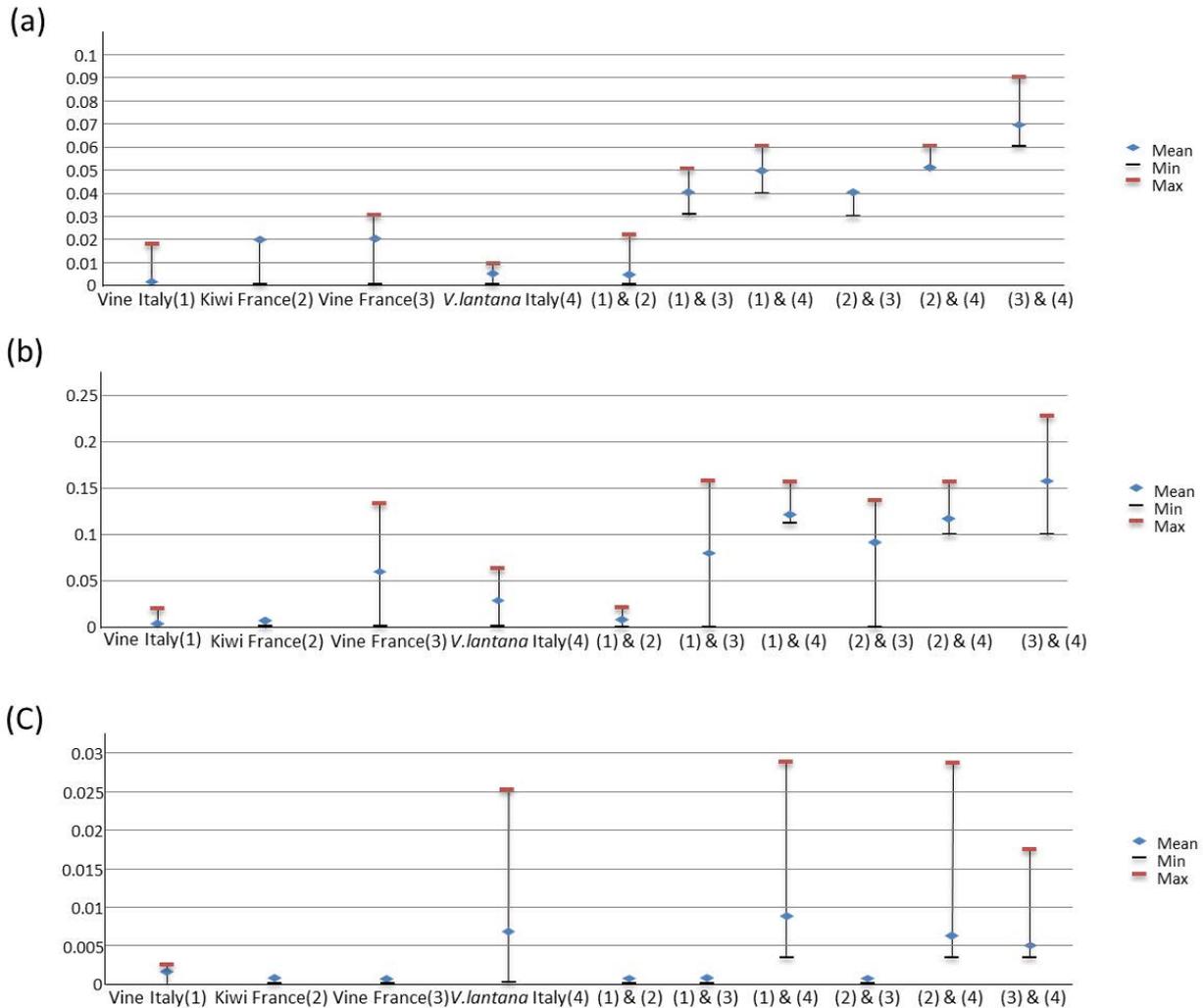


FIGURE 6: K2P genetic distances within and between the four populations of *Phytoseius finitimus* considered for the three molecular markers used (a) 12S rRNA, (b) Cytb mtDNA and (c) ITSS.

Morphological analyses.

Some statistical differences are observed for twelve continuous characters (Table 5). However, the statistical groups show that measurements of specimens from *V. lantana* are usually not different from specimens from the other three populations. The most frequent significant differences are observed between specimens from Corsica (vine and kiwi) and from Italy (vine and *V. lantana*). On the two axes of the multifactorial analysis explaining 47 % of the variation (Figure 7), specimens collected on *V. lantana* are close to those collected on vine-

Italy, these two populations being quite well separated from the specimens collected in Corsica, both on vine and kiwi. The variation within and between populations from Italy is lower than that observed within and between populations from Corsica. Mean differences in seta length even if statistically significant are however usually low. The most important difference is observed in *s6* length, this setae being significantly shorter for specimens collected on *V. lantana* (mean = 81 μm) than for specimens of the three other populations (88, 89, and 94 μm for mites collected on vine in Italy, on kiwi in Corsica and on vine in Corsica, respectively).

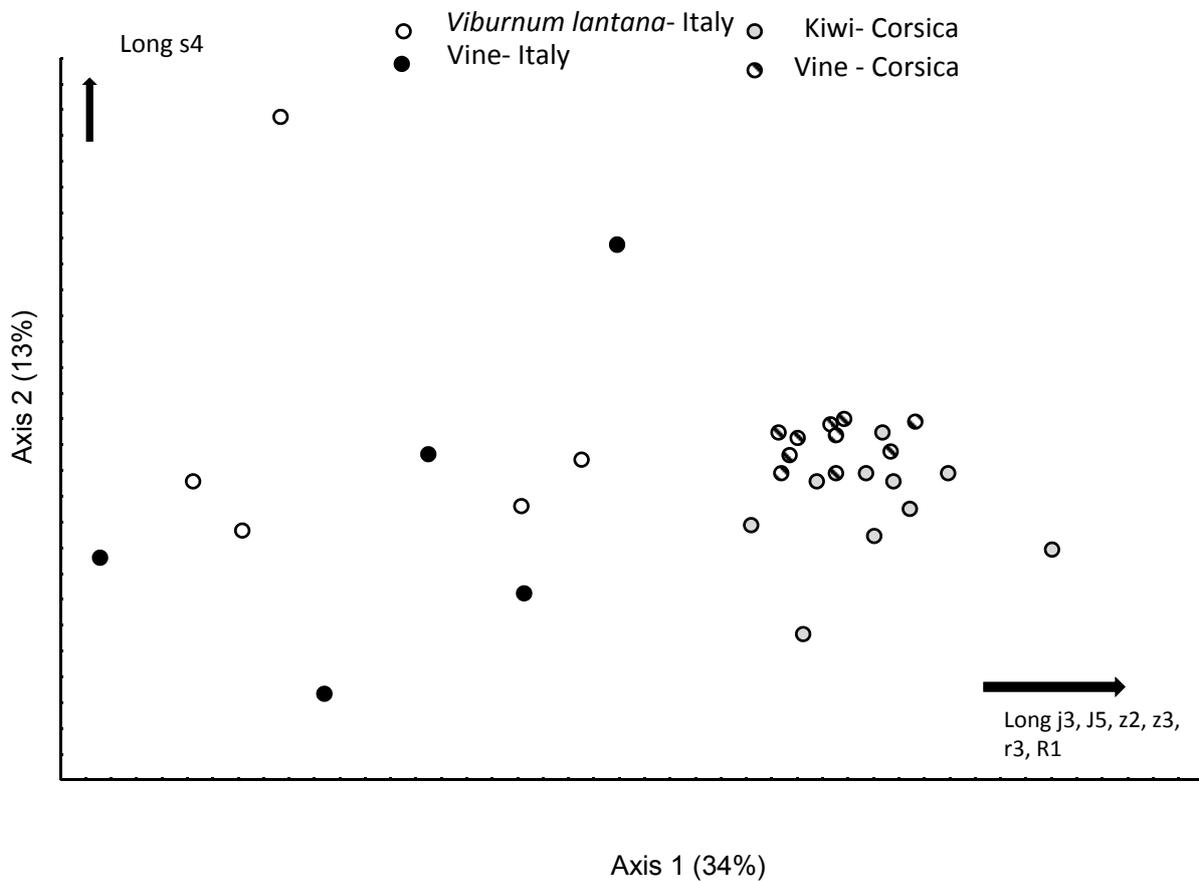


FIGURE 7: Scatter plots (PCA analysis) of the first two axes for seventeen morphological characters of specimens collected on kiwi in Corsica, *Viburnum lantana* in Italy, vine in Italy and vine in Corsica.

However the absolute value between these seta lengths, ranging between 7 and 13 μm , is according to the modelling of intraspecific seta length range proposed by Tixier (2012, 2013) included in intraspecific variation (Table 4). The $r\text{CL}_{95}$ calculated according to Tixier (2012) shows that most setae have a variation around the mean lower than 20 %, except for the smallest setae. This result is in accordance with results of Tixier (2012) when modelling the intraspecific variation rate around the mean for setae length of Phytoseiidae dorsal shield. These modelled variation intervals around the means (Tixier 2012) include the variation range of specimens herein studied for all seta considered.

Furthermore observations of shapes of spermatheca (Figure 8) as well as ventrianal shield

chaetotaxy and shape and peritremal extremity (reaching z2) do not reveal differences between the populations considered.

DISCUSSION

This study shows (i) the difficulty in interpreting DNA variation for species diagnosis and (ii) the necessity of an integrative taxonomic approach. Clear conclusion can be proposed for two clades (clades I and II) among the three observed on the mitochondrial DNA trees. Because (i) these two clades were not separated using the nuclear ITSS markers, (ii) distance overlap was observed for all markers (nuclear and mitochondrial) between intra and inter-population distances, (iii) these populations belong to a same group with the ABGD method

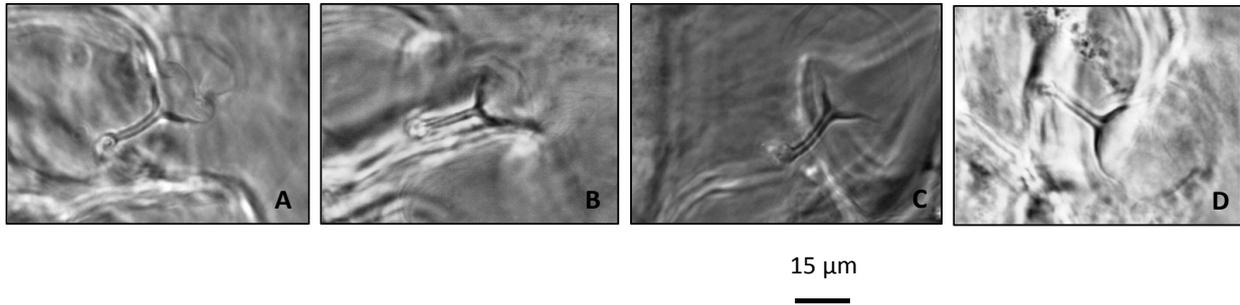


FIGURE 8: Spermatheca of the four populations of *Phytoseius finitimus*: A – kiwi-Corsica; B – *Viburnum lantana*-Italy, C – vine-Italy; D – vine-Corsica.

(using P distance), one specimen of vine-Corsica is included in the clade containing specimens from vine-Italy and kiwi-Corsica and (iv) the higher inter-population distances were much lower than the already observed ones between two species of the same genus, the convergent decision was that these two clades belong to the same species. However, for the status of the third clade containing specimens collected on *V. lantana*, the decision was more difficult. Because of (i) the well-separated clade including those specimens with both mitochondrial and nuclear markers, (ii) the high distances between those specimens and specimens from the three other populations, (iii) no clear overlap between sequences of these two groups for the 12S rRNA fragment and (iv) results obtained based on mtDNA fragments with the ABGD approach, two hypotheses could be proposed: (i) specimens of the four populations considered belong to a same species characterised by high molecular polymorphisms, or (ii) specimens from *V. lantana* belong to a species very close to the specimens of the three other populations. The final decision was made based on the observation of morphological characters. No difference between seta lengths of the four populations is observed, suggesting that they belong to the same species.

According to the markers and analytic concepts considered, diagnosis conclusion can be different and decision hard to dress.

Molecular markers and pitfalls.

Mitochondrial markers showed much more variation than was observed with the nuclear marker.

ITSS and mitochondrial markers provide thus different information; it is not possible based only on trees to give a clear decision on the state of clades obtained (different species or populations). Such a result was observed for other Phytoseiidae species for example indicating possible cryptic species within *Typhlodromus* (*Typhlodromus*) *pyri* Scheuten, *Neoseiulella aceri* and *Neoseiulus californicus* (Kanouh *et al.* 2010a; Okassa *et al.* 2011; Tixier *et al.* 2012) as well for other groups of mites in Mesostigmata (Roy *et al.* 2010), Ixodidae (Leo *et al.* 2010); Hydrachnidia (Stalstedt *et al.* 2013), Oribatida (Rosenberger *et al.* 2013; Kreipe *et al.* 2015), Tetranychidae (Navajas and Boursot 2003; Ros *et al.* 2008), Eriophyidae (Skoracka and Dabert 2010), especially for the COI mtDNA marker.

Such mitochondrial polymorphism can be due to the nature of molecule evolution (*i.e.* mtDNA is more sensitive to population bottlenecks, the absence of recombination in mtDNA) and to ecological features (*i.e.* dispersal, fecundity) that lead to population separations. Furthermore, Phytoseiidae mites are haplodiploid. Even if there are few studies on the impact of such features on mtDNA variation outside of Hymenoptera, Lohse and Ross (2015) suggest that this characteristic can imply a higher introgressions for mitochondrial compared to nuclear markers; they report current incongruence between nuclear and mitochondrial trees in haplodiploid organisms, including spiders.

As a consequence, some artificial groups can be obtained, underlying the necessity to use an integrative taxonomical approach, combining morpho-

logical and molecular characters as well as different DNA fragments and associated conceptual analyses (*i.e.* Dayrat 2005).

Which analytic methods to use?

This study first stresses the difficulty in interpreting phylogenetic trees. The same clades were obtained for the three algorithms used (Neighbour-Joining, Parsimony and Bayesian analyses), suggesting that at low taxonomic level the Neighbour-Joining tree provides the same clustering as the other model-based analyses (Holder and Lewis 2003; Yang and Rannala 2012). In addition the concatenated analysis of the three DNA markers does not provide a clearer answer as the same clusters were obtained as those with the two mitochondrial markers. The concatenated tree thus can hide some information as the signal obtained clearly depends on the resolution level of each DNA marker considered.

This study also stresses the necessity of having clear distance references for comparison. The ABGD aims to construct its own rules based on a data set (without *a priori* hypothesis on taxon status) (Puillandre *et al.* 2012a). This method overcomes common threshold applications, reported to be variable according to taxa considered (*i.e.* Collins and Cruickshank 2013). For the present dataset, more than one species are emphasized using the two mitochondrial markers. Four and five groups are even observed using the JC69 and K80 distances, suggesting (i) the high sensitivity of this clustering method and (ii) the impact of the number of sequences analysed (Puillandre *et al.* 2012b). Furthermore, even with the ABGD analysis based on P distance, the specimens from *V. lantana* were always assumed to belong to a different species. However using the ITSS marker, the results converge with other analyses, especially morphology suggesting that all specimens belong to a same species. The number of populations and sequences were quite low which can lead to artificial groups significantly detected by ABGD approach (Lhose 2009; Puillandre *et al.* 2012b). To our knowledge, this latter method has never been applied for the markers 12S rRNA, CytB mtDNA and only one time for ITS2 (Schwarzfeld and Sperling 2015); it has been essentially used for

the traditional barcode fragment (COI mtDNA) and in some rare cases for 16S rRNA (*i.e.* Puillandre *et al.* 2012b; Jörger *et al.* 2012; Guarnizo *et al.* 2015; Han *et al.* 2016). Results herein obtained can be due to the fact that other DNA fragments were considered and prior parameters not adapted. Furthermore, it was also carried out only once on mites, on feather mites using COI (Doña *et al.* 2015) making conclusions of ABGD relevance for mite species delimitation difficult.

This study contributes to the characterization of decision rules for molecular diagnoses within Phytoseiidae and more generally speaking within mites. It is the first time that molecular variation of a species of the subfamily Phytoseiinae is studied. Its variation is higher than already observed within species of the other two subfamilies (Typhlodromiinae and Amblyseiinae). Testing additional species of the sub-family Phytoseiinae would be interesting for understanding how subfamily and associated biological/ecological features can impact intraspecific variations (*i.e.* low dispersal, small population size). The present genetic distances are the highest ever observed within a Phytoseiidae species. Yet, it seems that genetic distances higher than 9 %, 23 % and 2.8 % would correspond to interspecific distances for the markers 12S rRNA, CytB mtDNA and ITSS, respectively. Those results are quite difficult to compare with those obtained for other groups, as the threshold can be different according to taxonomic entities (*i.e.* Collins and Cruickshank 2013). Furthermore, CytB mtDNA and 12S rRNA markers have been rarely used for species diagnosis within other mite groups. A recent study on ticks using the 12S rRNA markers proposes a minimal interspecific distance of 13.2% and an average intraspecific distance of 1.8%; no data is provided on maximal intraspecific distance (Lv *et al.* 2014).

These simple comparisons need to be completed with statistical analyses to determine the validity (and associated error) of a barcoding decision depending on the distance obtained between two sequences and to their proximity with the lower limit of intraspecific variation. Furthermore, when comparing with literature, these rules seem to be different depending on the genus as minimal genetic dis-

tance of 4 % for the 12S rRNA is observed between two close species (*P. persimilis* and *P. macropilis*). However, in this latter case, it is worthwhile to test if: (i) this low intraspecific distance is due to particular biological features of species of the atypical genus *Phytoseiulus* within Phytoseiidae, or (ii) *P. macropilis* and *P. persimilis* are synonyms and a 12S rRNA distance of 4 % would correspond to an intraspecific value, as for all the Phytoseiidae species tested until now.

Implications for biological control?

Finally, for biological control matters, further experiments should be planned to determine whether the present mitochondrial clades show different behaviour and biological features. Tixier *et al.* (2010a) observed that mitochondrial clades within the species *Phytoseiulus longipes* Evans were associated with different feeding abilities (prey preferences). The closest populations with molecular markers correspond to specimens collected in cultivated areas (vine-Italy and -Corsica and kiwi-Corsica) but in geographically distinct areas. The well-differentiated population was collected from uncultivated plants (*V. lantana*) nearby the vine-Italy plot. It is interesting to note that morphological analyses do not show the same similarities, as morphological similarity seems more associated with geographical distances than with plants and cultivated situation. Further investigations would be to determine how selection pressures associated with agricultural practices are linked to such a differentiation for assessing the consequences on colonisation of specimens of *P. finitimus* dispersing from uncultivated vegetation towards crops.

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