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Increased molecular divergence of two endemic *Trachelipus* (Isopoda, Oniscidea) species from Greece reveals patterns not congruent with current taxonomy

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In the present study, we employed three mitochondrial DNA genetic markers in a phylogenetic analysis aiming at the delineation of the relationships amongst nominal *Trachelipus kytherensis* populations, as well as between populations of this species and of *Trachelipus aegaeus* and a new form, occurring syntopically with the latter. Both the phylogenetic analysis and the genetic distances separating populations, show the presence of several distinct and well differentiated clades that undermine the monophyly of *T. kytherensis*. On the other hand, despite the insular distribution of *T. aegaeus* populations, their divergence is low and the monophyly of this taxon can be rescued by the inclusion of two more insular populations previously assigned to *T. kytherensis*. The patterns of genetic divergence among clades are only partially congruent with the geographic distribution of populations. The validity of taxonomic characters used so far in the genus appears to be questionable; therefore, a more comprehensive phylogenetic study at a population level is deemed necessary for understanding the divergence of *Trachelipus* lineages. *Biological Journal of the Linnean Society*, 2008, **95**, 361–370.

ADDITIONAL KEYWORDS: 12S rRNA – 16S rRNA – Aegean – CO *I* – Peloponnisos.

INTRODUCTION

The phylogenetic relationships among terrestrial isopod species are still largely unknown because robust analyses have started to appear only relatively recently (Erhard, 1998a, b; Michel-Salzat & Bouchon, 2000; Mattern & Schlegel, 2001; Wetzer, 2001, 2002; Mattern, 2003). These analyses have focused mainly on the phylogeny of higher taxonomic groups inside the order Isopoda or the suborder Oniscidea. A few attempts towards a resolution of relationships among congeneric species or populations of nominal species have been made (Cobolli Sbordoni *et al.*, 1995; Sbordoni *et al.*, 1997; Rivera *et al.*, 2002; Charfi-Cheikrouha, 2003; Klossa-Kilia *et al.*, 2006), even though the available data indicate large levels of genetic divergence among populations, and some extent of incongruence between the patterns obtained from molecular data and the morphological delineations of taxa. Species-level taxonomy has been based mainly on a few secondary sexual characters of males, although recent analyses based on molecular markers have indicated that species definitions based

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on morphology may underestimate the true levels of divergence among populations (Klossa-Kilia, Kilias & Sfenthourakis, 2005; Klossa-Kilia *et al.*, 2006). Furthermore, within several genera or species groups, morphological characters do not provide clear-cut taxonomic resolution, so that many changes in the interpretation of nominal species have appeared in the literature (Schmalfuss, 2003, 2004).

In a previous work (Klossa-Kilia *et al.*, 2006), we studied the genetic structure of populations belonging to the genus *Ligidium* Brandt, 1833 represented in Greece by highly specialized forms that occur only in a narrow range of riparian habitats. Unexpected levels of genetic divergence and patterns of differentiation were found. In the present study, we focus on populations of a less stenoecious species (*Trachelipus kytherensis* Strouhal, 1929) that exploits humid habitats, mainly in forested sites, being more abundant near freshwater but not restricted therein. This species is endemic to Greece and has a relatively broad distribution. It can be found from sea-level to mountaintops. Relatively dry habitats, such as agricultural land, scrubs, and dry woodland, constitute a significant barrier between its populations. The current diagnosis of the species is not based on unequivocal characters because it shows striking resemblance to another Greek species (*Trachelipus aegaeus* Verhoeff, 1907) with an allopatric distribution. The nomenclature of *T. kytherensis* has changed in time, according to presumed synonymy of previously described species. The most recent view is that the species *T. kytherensis* is distributed in Crete, Kythira island and the Greek mainland, whereas the similar species *T. aegaeus* is present on central Aegean islands and a few Aegean coastal parts of the Greek mainland (Schmidt, 1997). A correct updated description of the species distributions is provided elsewhere (Schmalfuss, 2004).

An investigation of the genetic variation at population level was considered necessary for the clarification of both the taxonomical issues and the actual patterns of divergence within this species' group. A broader phylogenetic analysis of the genus *Trachelipus* Budde-Lund, 1908 cannot be meaningful without such clarifications at the population level. Indeed, according to current taxonomy (Schmalfuss, 2003, 2004), the genus *Trachelipus* includes some 50 species distributed all around the Palearctic, with eight species recorded from Greece, four of which are endemic to the country. More specifically, besides the afore-mentioned species, the species present in Greece are *Trachelipus arcuatus* (Budde-Lund, 1885), *Trachelipus buddelundi* (Strouhal, 1937), *Trachelipus camerani* (Tua, 1900), *Trachelipus cavaticus* (Schmalfuss, Paragamian & Sfenthourakis, 2004), *Trachelipus razzautii* (Arcangeli, 1933), and *Trachelipus squamuliger* (Verhoeff, 1907). Of these, *T. kytherensis*, *T. aegaeus*, *T. buddelundi*, and *T. cavaticus* are Greek endemics, with *T. kytherensis* also being the most widely distributed species of the genus in the country. *Trachelipus buddelundi* is known only from its original description from the north-east Aegean island of Chios and its validity is questionable, whereas *T. cavaticus* is restricted to the island of Crete, inhabiting mainly caves. The other species have been recorded in relatively few sites at the north-northwestern parts of Greece (two have also been recorded from Lesvos Island in the north-east Aegean). The validity of these morphologically defined species, though, should be evaluated on the basis of detailed molecular studies.

In the present study, we employed three mitochondrial (mt)DNA genetic markers in a phylogenetic analysis aiming to delineate the relationships amongst nominal *T. kytherensis* populations, as well as between populations of this species and populations of *T. aegaeus* and a new form, occurring syntopically with one population of the latter species.

MATERIAL AND METHODS

Populations studied

We sampled 14 populations of the nominal *T. kytherensis* from several sites scattered within its overall known range (Fig. 1; Table 1). To clarify the relationships with the morphologically similar *T. aegaeus*, we also collected material from three populations of this species within its known distributional range (Fig. 1; Table 1). In the course of sampling, we found a new form, clearly distinct from the above mentioned species on morphological grounds, occurring syntopically with one population of *T. aegaeus* on an island near the borderline between the distributional ranges of *T. aegaeus* and *T. kytherensis*. The same new form was found also on a tiny nearby islet from where *T. aegaeus* is missing. Therefore, we also included material (two populations) of this form (*Trachelipus* **sp. nov.**; under formal description) to obtain a better resolution of relationships between all these populations.

DNA extraction and PCR amplification

Most of the specimens used in the analysis were preserved in absolute ethanol and were collected during 2005–2006. However, there was one population whose specimens were collected in 1992 and were preserved in 70% alcohol (Table 1). Total DNA extraction (using part or the whole animal) was carried out using the Macherey–Nagel Tissue kit following the manufacturer's protocol. DNA was ultimately extracted from 67 *T. kytherensis* specimens (14 popu-

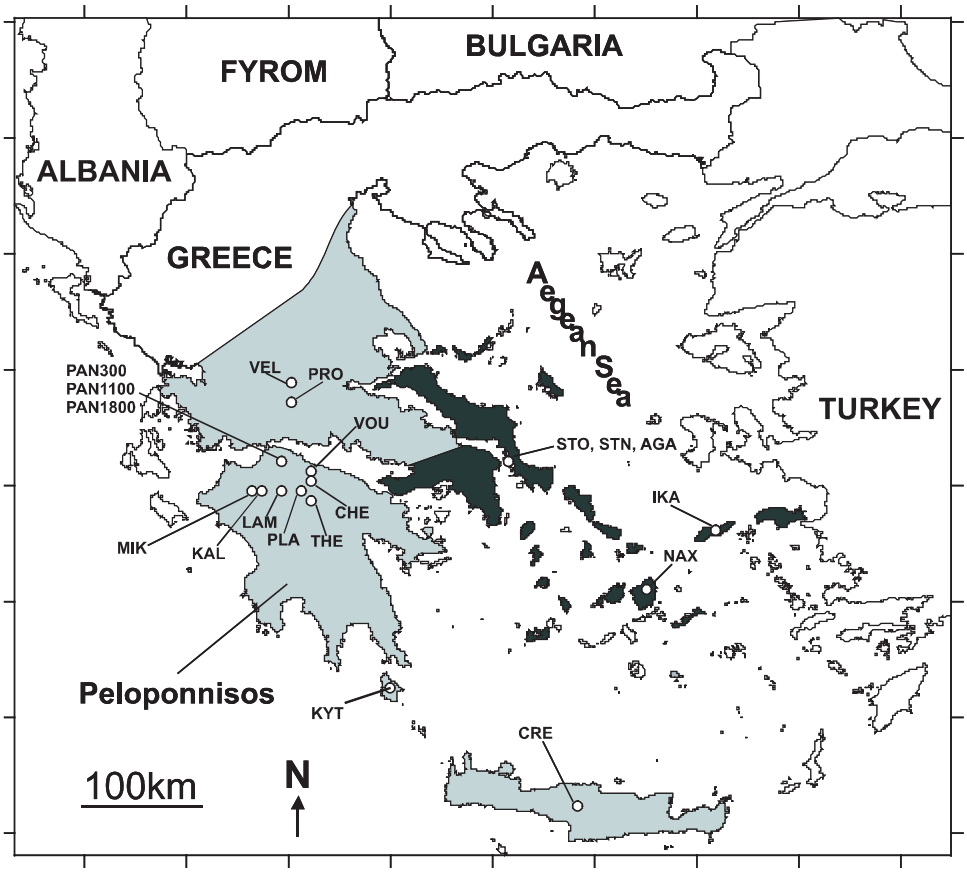

Figure 1. The distribution of nominal *Trachelipus kytherensis* (light grey) and *Trachelipus aegaeus* (dark grey). Population map codes are as presented in Table 1.

lations; Table 1), six *T. aegaeus* specimens (three populations), and four *Trachelipus* **sp. nov.** (two populations). For each one of the specimens, we produced the sequence of a partial segment of three mtDNA genes [16S rRNA, 12S rRNA and cytochrome oxidase subunit *I* (CO *I*)], using the polymerase chain reaction (PCR) technique (Saiki *et al.*, 1988). PCRs were carried out as described previously (Klossa-Kilia *et al.*, 2006).

For the 16S rRNA gene, the widely applicable primers 16Sar and 16Sbr (Palumbi, 1996) were used along with one newly-designed forward primer, namely 16Sar-int-sf (5′-GCC GCA GTA THC TRA CTG TGC T-3′). The produced amplicons were 400–450 bp in length. The primers 12SCRF and 12SCRR (Hanner & Fugate, 1997) were used for the amplification of 12S rRNA, producing a fragment of 350 bp in length. Finally, for the amplification of the CO *I* gene, the primers COI-F and COI-R (Folmer *et al.*, 1994) were used for most of the specimens. However, we also designed and used two additional CO *I* primers, internal to the previous ones. The new primers were COIFint: 5′-GGG ACA GCH CTK AGV RTA AT-3′ and COIRint: 5′-GCY CCY GCY AAW ACA GGK ARD GA-3′. The targeted CO I segment was 500–550 bp in length depending on the primer pair used.

PCR products were purified using commercially available spin columns (Macherey–Nagel). Individual sequences were determined via automated sequencing of the forward strand of each mtDNA gene segment. The primers in the sequencing reactions were as those described in the amplification procedure. All sequences determined in the present study have been deposited in GenBank under the accession numbers reported in Table 1.

Phylogenetic and sequence data analysis

For each mtDNA sequence data set, multiple-sequence alignments were performed with CLUSTALW (Thompson, Higgins & Gibson, 1994) of the ClustalW Service at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw) using the default parameters. The computer-generated alignment was further adjusted manually. Additionally, the

Table 1. List of populations of *Trachelipus kytherensis* used in the analysis, populations' (map) codes and GenBank accession numbers of sequences originating from each population

Species name	Population	Population map code	Acc. No. COI (*n*)	Acc. No. 16S rRNA (*n*)	Acc. No. 12S rRNA (*n*)
Trachelipus kytherensis	1. Velouchi Mt – 1200 m, Bagasaki, Sterea Ellada	VEL	EF027452–EF027453 (2)	EF027526–EF027527 (2)	EF027601–EF027602 (2)
Trachelipus kytherensis	2. Proussos – 600 m, Sterea Ellada	PRO	EF027447–EF027451 (5)	EF027521–EF027525 (5)	EF027596–EF027600 (5)
Trachelipus kytherensis	3. Panachaiko Mt – 300 m, Peloponnisos	PAN300	EF027435–EF027436 (2)	EF027507–EF027508 (2)	EF027582–EF027583 (2)
Trachelipus kytherensis	4. Panachaiko Mt – 1100 m, Peloponnisos	PAN1100	EF027434 (1)	EF027505 (1)	EF027580 (1)
Trachelipus kytherensis	5. Panachaiko Mt – 1800 m, Pelloponnisos	PAN1800	EF027428–EF027433 (6)	EF027498–EF027503 (6)	EF027573–EF027578 (6)
Trachelipus kytherensis	6. Vouraikos – 500 m, Peloponnisos	VOU	EF027387–EF027398 (12)	EF027457–EF027468 (12)	EF027532–EF027543 (12)
Trachelipus kytherensis	7. Chelmos Mt – 900 m, Peloponnisos	CHE	EF027399–EF027404 (6)	EF027469–EF027474 (6)	EFO27544–EF027549 (6)
Trachelipus kytherensis	8. Theoktisto, Peloponnisos	THE	EF027443–EF027446 (4)	EF027515, EF027518–EF027520 (4)	EF027590, EF027593–EF027595 (4)
Trachelipus kytherensis	9. Planiterou, Chelmos Mt, Peloponnisos	PLA	EF027437–EF027442 (6)	EF027509–EF027514 (6)	EF027584–EF027589 (6)
Trachelipus kytherensis	10. Lambeia, Peloponnisos	LAM	EF027416–EF027421 (6)	EF027486–EF027491 (6)	EF027561–EF027566 (6)
Trachelipus kytherensis	11. Kalentzi, Peloponnisos	KAL	EF027405–EF027407 (3)	EF027475–EF027477 (3)	EF0274550–EF27551 (3)
Trachelipus kytherensis	12. Mikrouleika, Peloponnisos	MIK	EF027422–EF027427 (6)	EF027492–EF027497 (6)	EF027567–EF027572 (6)
Trachelipus kytherensis	13. Kythira Island	KYT	EF027412–EF027415 (4)	EF027482–EF027485 (4)	EF027557–EF027560 (4)
Trachelipus kytherensis	14. Crete	CRE	EF027408–EF027411 (4)	EF027478–EF027481 (4)	EF027553–EF027556 (4)
Trachelipus aegaeus	15. Naxos Island	NAX	EF659958–EF659960 (3)	EF659966–EF659968 (3)	EF659948–EF659950 (3)
Trachelipus aegaeus	16. Nas, Ikaria Island*	IKA	–	EF659972–EF659973 (2)	EF659954–EF659955 (2)
Trachelipus aegaeus	17. Stouronisi islet, southern Evvoikos gulf	STO	EF659961 (1)	EF659969 (1)	EF659951 (1)
Trachelipus **sp. nov.**	18. Stouronisi islet, southern Evvoikos gulf	STN	EF659962–EF659963 (2)	EF659970–EF659971 (2)	EF659952–EF659953 (2)
Trachelipus **sp. nov.**	19. Agios Andreas islet, southern Evvoikos gulf	AGA	EF659956–EF659957 (2)	EF659964–EF659965 (2)	EF659946–EF659947 (2)
Outgroups					
Armadillidium lobocurvum		ALO	EF027702	EF027628	EF027664
Tylos ponticus		TPO	EF027456	EF027531	EF027606

In the accession numbers columns, numbers in parenthesis (*n*) indicate the number of sequences produced from each population and each genetic marker.
*Specimens preserved in 70% alcohol, collected in 1992.

alignment of the CO *I* data set was verified against published CO *I* sequences of other isopod species available in GenBank. The CO *I* sequences produced in the present study were unambiguously aligned to the retrieved CO *I* sequences, whereas no gaps and/or stop codons were present. Therefore, the authenticity of the produced mtDNA CO *I* sequences was verified. Pairwise genetic distances were estimated using MEGA, version 3.1 (Kumar, Tamura & Nei, 2004) and the Kimura two-parameter model (Kimura, 1980). In all phylogenetic analyses, we used sequences of *Tylos ponticus* Grebnicky, 1874 and *Armadillidium lobocurvum* Verhoeff, 1902 as outgroups produced for the present study (Table 1). These species were used as outgroups because the phylogenetic relationships between genera and families inside Oniscidea are not known at an adequate resolution level. The use of these two genera cover a broad range of possible clade proximities. At the same time, available sequences for oniscidean species are limited to a few taxa (e.g. Ligiidae, Trichoniscidae, Porcellionidae), most of which are not known to be closer relatives (compared with Armadillidiidae) to Trachelipodidae. In addition, Trachelipodidae almost certainly is not a monophyletic taxon, so the use of other genera currently assigned to this family does not solve the problem of proper outgroup selection.

Separately, and for each mtDNA gene segment, we performed a preliminary maximum parsimony (MP) analysis (with the same parameters as those described below). In this analysis, all individuals for whom sequence data was available were included. To examine whether the sequences from the three mtDNA genes could be combined in a single analysis, a partition-homogeneity test (Farris *et al.*, 1995) was run in PAUP* (Swofford, 2002), and significance was estimated with 1000 repartitions. In almost all cases, the individuals originating from the same population would either have identical sequences or would cluster together with bootstrap support value higher than (or equal to) 90% (results not shown). Furthermore, the topology of the produced MP trees, from each one of the mtDNA gene segments, was congruent for the most part, but the bootstrap support in some of the nodes was below 50% (results not shown). Therefore, to optimize computation time, one subset of sequences was compiled by choosing sequences for a maximum of two to three individuals from each sampling locality to represent the entire population. The partition-homogeneity test (Farris *et al.*, 1995) indicated conflicting phylogenetic signals between the data sets ($P = 0.01$). However, the conflict could evidently be attributed to the topology of the terminal branches, which varied depending on the phylogenetic marker used. Because all major clades were present in all three phylogenetic trees (12S rRNA, 16S rRNA and CO *I* trees) and aiming in an optimally resolved phylogeny, the three mtDNA segments were combined to a single concatenated data set. The final subset used in all subsequent phylogenetic analyses consisted of the sequences of 36 nominal *T. kytherensis*, three *T. aegaeus*, three *Trachelipus* **sp. nov.** specimens and one specimen for each of the two outgroup species. To distinguish phylogenetic signal from random noise, the final subset was submitted to the test described in (Hillis & Huelsenbeck, 1992) as implemented in PAUP* (Swofford, 2002). The g_1 statistic pointed to a tree-length distribution strongly skewed to the left, an indication of high phylogenetic content in the dataset. Following that, phylogenetic analysis performed on the above mentioned compiled subset of sequences involved MP, Bayesian inference (BI) and maximum likelihood (ML) analyses.

The MP analysis was performed using PAUP* (Swofford, 2002), with heuristic searches using stepwise addition of sequences and performing tree-bisection-reconnection branch swapping (Swofford *et al.*, 1996). Gaps were treated as missing characters. Several schemes of MP analyses were performed, including differential weighting of transversions and transitions and exclusion of the third codon position of the CO *I* sequences.

The BI analysis was performed in MrBayes3.1 (Ronquist & Huelsenbeck, 2003) and a different substitution model was applied in each mtDNA gene segment. The substitution models implemented in the analysis were those suggested by MODELTEST, version 3.7 (Posada & Crandall, 1998) according to the Akaike Information Criterion (AIC) (Akaike, 1974). The number of generations was set to 3×10^6. The average SD of split frequencies of the two simultaneous and independent runs (four chains implemented in each run) performed by MrBayes, reached stationarity well before 3×10^5 generations. A tree was sampled every 100 generations and, consequently, the summaries of the Bayesian inference relied on 60 000 samples (from two runs). From each run, 22 501 samples were used, whereas 7500 were discarded as burn-in phase. A consensus tree was constructed by MrBayes3.1 from the remaining 45 002 trees. Support for nodes was assessed with the posterior probabilities of reconstructed clades as estimated by MrBayes3.1 (Ronquist & Huelsenbeck, 2003).

For ML analysis, we used GARLI, version 0.951 (http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html) (Zwickl, 2006), which performs phylogenetic searches on aligned nucleotide datasets using the ML criterion. In this analysis, the three mtDNA gene segments were analysed as a single evolving fragment. The model of nucleotide substitution implemented in the analysis, was the GTR+G+I (Rodriguez

et al., 1990), as suggested by MODELTEST (Posada & Crandall, 1998) under the AIC criterion. Bootstrap support for the ML analysis was assessed by 100 replicates and the 50% majority-rule consensus tree was constructed using PAUP* (Swofford, 2002).

Kimura two-parameter (Kimura, 1980) genetic distances were used in various comparisons and were calculated using MEGA, version 3.1 (Kumar *et al.*, 2004).

RESULTS

Sequence data analysis

The attempt to amplify all three gene segments for each individual was successful in almost all cases. We have not been able to produce the CO *I* sequences for the specimens from Ikaria Island. However, to investigate the relationship of the samples from Ikaria Island with the remaining ones, we included them in the analysis coding the unavailable CO *I* fragment as missing data. For all three mtDNA gene segments, we determined the sequences of 77 (75 for CO *I*) *Trachelipus* specimens plus the two outgroup species, *A. lobocurvum* and *T. ponticus* (Table 1). Alignment-ambiguous regions were not evident in any of the data sets; therefore, no sequence data were omitted from subsequent analysis.

Phylogenetic analyses

The alignment of the compiled subset of sequences contained 1276 nucleotides. Of these, 564 (44%) were variable and 408 (32%) were parsimony informative. The net average genetic distance between *Trachelipus* species ranged from 7.2 (*T. kytherensis* – *T. aegaeus*) to 10.5% (*Trachelipus* **sp. nov.** – *T. kytherensis*). The net average genetic distances separating ingroup from outgroup species were in the range 7.7–12.2%.

The best-fit models selected by MODELTEST, version 3.7 (Posada & Crandall, 1998) were the TrN+G (base frequencies: A = 0.3745, G = 0.1519, C = 0.1404, T = 0.3332, shape parameter $\alpha = 0.2978$), TVM+I+G (base frequencies: A = 0.2512, C = 0.1530, G = 0.2484, T = 0.3474, shape parameter $\alpha = 0.5160$, pinvar = 0.5000), and HKY+G (base frequencies: A = 0.3139, C = 0.2008, G = 0.1453, T = 0.3401, shape parameter $\alpha = 0.2831$) for the 16S rRNA, CO *I* and 12S rRNA data partitions, respectively.

All three phylogenetic methods (MP, BI, and ML) produced trees with quite congruent topologies. The few differences observed between the different methods were mostly related to terminal branch swapping. The different phylogenetic schemes applied in the MP analyses, produced trees of marginally different topology. Consequently, in the MP analysis, we present the results of the most straightforward one. The 50% majority-rule consensus tree, produced by the Bayesian analysis, is shown in Figure 2. Besides the posterior probabilities of each clade the bootstrap values of the MP and ML analysis are also presented. In this tree, it is evident that there are six different *Trachelipus* clades. However, the relationships between these clades are not unambiguously defined because the statistical support in some cases is low. Nevertheless, in the phylogenetic tree shown in Figure 2, the first-formed ingroup clade (C1) incorporates the samples from the islets of Agios Andreas (AGA) and Stouronisi (STN), belonging to the new undescribed form (*Trachelipus* **sp. nov.**). Following that, clade C2 includes Kythira (KYT), Naxos (NAX), Crete (CRE), and Stouronisi (STO – *T. aegaeus* specimen). Hence, clade C2 includes nominal populations of both *T. kytherensis* and *T. aegaeus*, whereas C1 is ambiguously related to clade C2 and the remaining *Trachelipus* clades. Following clade C2, there is clade C3 that involves a nominal *T. kytherensis* population from Velouchi Mt (VEL). This clade, in both the MP and the BI analyses, appears to be more closely related to clade C4 (see below), whereas, in the ML analysis, it is related more closely to clade C2. All remaining *T. kytherensis* populations are included in clades C4 and C5. Clade C4 consists of the samples from Vouraikos (VOU), two of the samples of Chelmos Mt (CHE), and the specimens from Proussos (PRO). The last clade, namely C5, contains only populations from Peloponnisos peninsula and can be divided into two subclades C5A and C5B. Subclade C5A includes all the populations of Panachaiko Mt (PAN300, PAN1100, PAN1800), the specimens from Lambeia (LAM), and the other two samples from Chelmos Mt (CHE), whereas subclade C5B contains the samples from the populations of Planiterou (PLA), Kalentzi (KAL), Mikrouleika (MIK), and Theoktisto (THE). The net average genetic distance between the four different *Trachelipus* clades (as computed with MEGA) was in the range 5.6–14.2%. The net average genetic distances (each mtDNA fragment separately) between the studied *Trachelipus* populations was well above 10% in the majority of the pairwise comparisons, whereas the maximum divergence was 19%, 20%, and 21% for the 12S rRNA, 16S rRNA, and the CO *I* genes, respectively (see Supporting Information, Tables S1, S2, S3).

DISCUSSION

In the present study, we present the first investigation into the relationships between nominal populations of *T. kytherensis* as well as between *T. kytherensis* and *T. aegaeus* populations. According to morphological criteria, the latter species is the one most closely related to *T. kytherensis* (Schmidt, 1997).

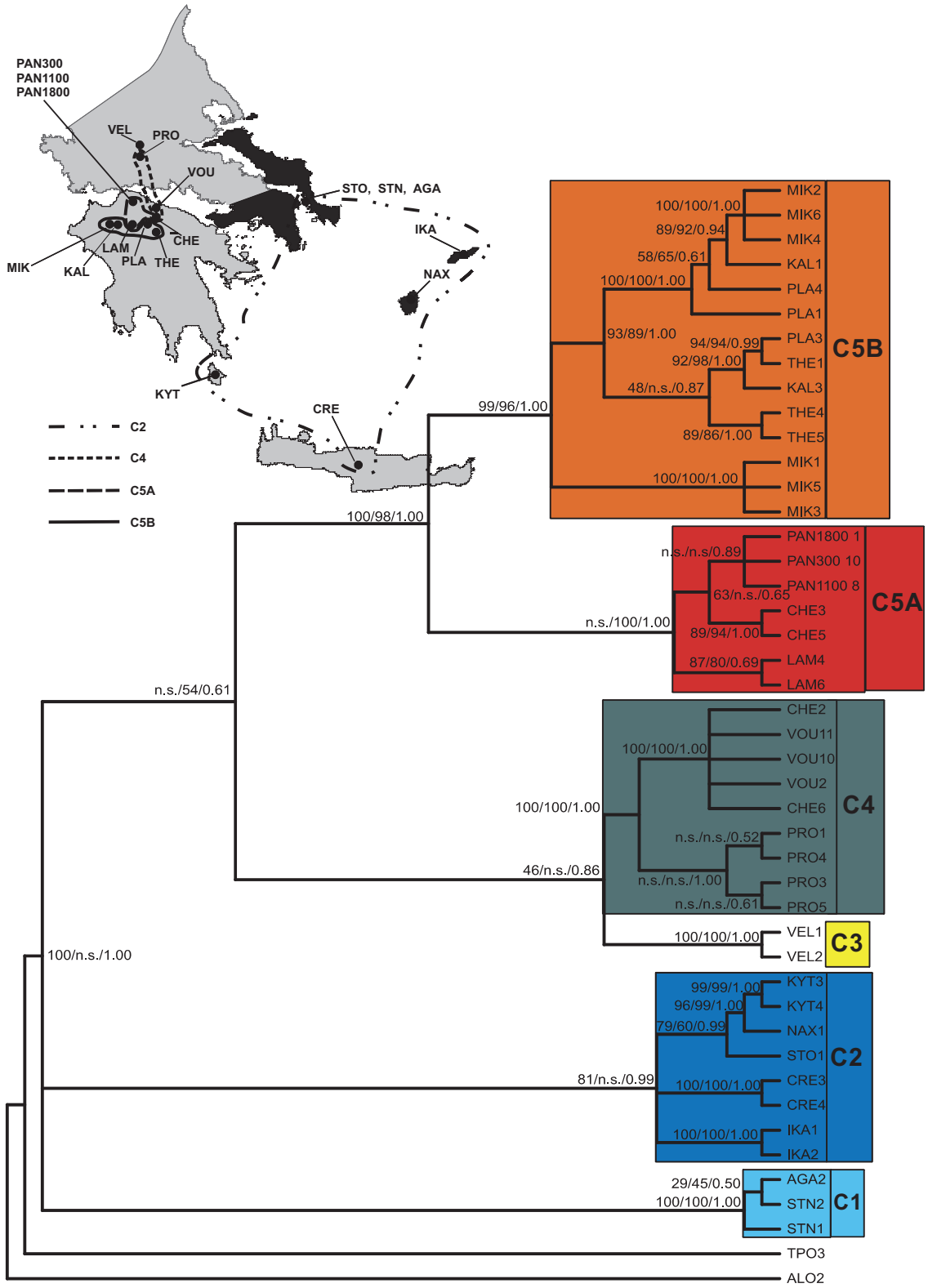

Figure 2. Fifty percent majority-rule consensus tree of the Bayesian Inference (BI) analysis of the Greek *Trachelipus* species. Numbers next to clades are the bootstrap support values of maximum parsimony (MP) and maximum likelihood (ML) analysis and the posterior probabilities of the BI analysis (MP/ML/BI). The distribution of each phylogenetic clade (except of those comprised of two or a single population) is depicted on the embedded map. Numbers on the left of the population's code refer to the individual sequence used in the analysis. Population map codes are as presented in Table 1.

Additionally, we aimed to assess the relationships of these two species with an undescribed *Trachelipus* form that was found to occur syntopically with *T. aegaeus*. The relationships of the populations were inferred with the use of three mtDNA markers, and reconstructed using three methods of phylogenetic analysis (MP, BI, and ML).

The relationships between the six (Fig. 2) different *Trachelipus* clades and subclades that were recovered congruently by all three phylogenetic methods are not adequately supported in all cases. However, the fact that the three different phylogenetic methods applied produced almost identical topologies is an indication that the underlying evolutionary history of the populations involved is roughly reflected in the phylogenetic tree of Figure 2.

Regardless of the relationships between clades, it is evident and strongly supported that some of the populations that are currently assigned to *T. kytherensis* are more closely related to populations of *T. aegaeus* (see clade C2) rather than to their conspecifics. The populations of *T. kytherensis* that are more closely related to *T. aegeaus* originate from the islands of Crete and Kythira. Furthermore, the populations (AGA, STN) of the new undescribed form do not show a robust relationship with either *T. kytherensis* or *T. aegaeus*. We could attribute the ambiguous position of this clade to the restricted number of *Trachelipus* species used overall. Hopefully, the future inclusion of more species distributed in the Greek territory will resolve the issue. At the same time, the population of Velouchi Mt (VEL), belonging to the nominal *T. kytherensis*, forms a clade that is loosely related to its conspecifics of clade C4. Furthermore, in the ML analysis, the population of Velouchi Mt appears to be more closely related to clade C2 (the insular clade), raising reasonable doubts about the taxonomic status of this population.

A closer look into clades C4 and C5 that include most of the nominal *T. kytherensis* populations reveals a phylogeographic pattern that is quite unexpected. Even though the populations included originate mostly from the northern part of the Peloponnisos peninsula, and are not lying very far apart, their phylogeographic pattern is highly-structured, as demonstrated by the branching pattern of the tree in Figure 2. Furthermore, the clustering of populations is only partially conforming to their geographical origin. The most striking example is the clustering of Vouraikos (VOU) and part of Chelmos (CHE) populations from northern Peloponnisos with that of Proussos (PRO) from central continental Greece. A similar grouping of populations originating from the same locations has also been found in the genus *Ligidium* (Klossa-Kilia *et al.*, 2006), indicating a possible historical connection between the northern slopes of Chelmos mountain (where Vouraikos river flows) with central continental Greece. The habitats at the mountain top probably acted as barriers for populations from the rest of the Peloponnisos peninsula. The situation in the stenoecious *Ligidium* is more clear-cut than in the somewhat more euryoecious *Trachelipus*, where the Chelmos population (CHE) also contains samples belonging to the Peloponnisos clade (C5). Because these animals can disperse through humid forests, there may have been a mixing of clades by dispersal through the extensive forests occurring in elevated sites of Chelmos Mt, which is not possible for the strictly riparian Greek species of *Ligidium*.

Another interesting result is the separation of the population from Velouchi Mt (VEL) from the one from Proussos (PRO) in distant clades. These sites lie a few kilometers apart and are not separated by any apparent barrier other than elevation (the population of Velouchi comes from 1200 m while the one of Proussos from 600 m a.s.l.). On the other hand, no elevational differentiation has been found in populations from Panachaiko Mt in the Peloponnisos clade.

The highly-structured phylogenetic tree and the lack of an overall geographic pattern in the clustering of *T. kytherensis* populations indicates that we are not dealing with a single species, but rather with several that are morphologically hard to distinguish, at least by means of the currently used morphological characters. This is further corroborated by the genetic distances separating the clades hosting nominal *T. kytherensis* populations. For example, the net average genetic distance between clades C4–C5A and C4–C5B is 13.9% and 14.2%, respectively. In general, it can be argued that the genetic distances recorded in the present study are quite large compared with those reported for different species and even genera in other studies of terrestrial isopods. For example Rivera *et al.* (2002) and Charfi-Cheikrouha (2003) report a maximum distance of 2.1% among populations of *Armadillidium pelagicum* Arcangeli, 1955 from Tunisia and distances ranging from 10.7% to 12.6% between this species and the well-defined separate species *A. album* Dollfus, 1887. Other well-defined species of isopods are reported to be separated by genetic distances (estimated from gene segments homologues to those used in the present study) in the range 13–28% (Rivera *et al.*, 2002; Baratti, Khebiza & Messana, 2004; McGaughran *et al.*, 2006). The estimated genetic distances separating different *Trachelipus* species, but also nominal *T. kytherensis* populations (see Supporting Information, Tables S1, S2, S3), fall within this range. At the same time, the monophyly of C2 clade is adequately supported, despite the relatively large genetic distances among some of the populations included in it, which could be

attributed to the large geographic distances separating them and to their isolation (i.e. because all of them live on islands).

In view of the above-mentioned patterns, the taxonomy of this group of species should be revised to a significant degree. Given the incomplete sampling of Greek *Trachelipus* populations in the present study, we can only provide tentative guidelines towards such a revision based on our most robust results. The strongly supported clade C2 monophyly indicates that the populations from the islands of Kythira and Crete should be transferred to *T. aegaeus* to maintain the monophyly of this species. This would mean that the name *T. kytherensis* is no longer valid because it was originally established for populations from Kythira Island (and *T. aegaeus* has priority). The original name *T. palustris* Strouhal, 1936, established for populations from Panachaiko Mt, synonymized with *T. kytherensis* by (Schmalfuss, 2003, 2004), should be restored for the strongly supported subclade C5A, possibly including also the whole Peloponnisos clade (C5). A more inclusive analysis, with more populations from central continental Greece, is needed to clarify the status of clades C3 and C4. Finally, our results provide further support for the description of the new form from Agios Andreas (AGA) and Stouronisi (STN) as a new species.

The molecular analyses provided in the present study show that the actual variation inside at least some of the currently established *Trachelipus* species is much larger than that revealed by morphological characters. A similar situation has also been found for Greek species of the isopod genus *Ligidium* (Klossa-Kilia *et al.*, 2006).

In summary, both the phylogeny presented here and the genetic distances separating populations appear to justify the necessity of further investigation into the phylogeny of the Greek *Trachelipus* species using a population by population approach. It is likely that morphology inadequately describes real variation inside and among species; hence, diagnoses based on the morphological characters used so far for the delineation of *Trachelipus* species should be reconsidered under the light of more extensive molecular phylogenetic analyses.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Average genetic distances between the studied *Trachelipus* populations based on the 12S rRNA sequences.

Table S2. Average genetic distances between the studied *Trachelipus* populations based on the 16S rRNA sequences.

Table S3. Average genetic distances between the studied *Trachelipus* populations based on the CO *I* sequences.

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