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Molecular phylogeny and phylogeography of the Greek populations of the genus *Orthometopon* (Isopoda, Oniscidea) based on mitochondrial DNA sequences

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We infer phylogenetic relationships among isopod species of the genus *Orthometopon* distributed in the Greek area, comparing partial mitochondrial DNA sequences for cytochrome oxidase I (COI). All phylogenetic analyses produced topologically identical trees that revealed a well-resolved phylogeny. These trees support the monophyly of the genus *Orthometopon*, and suggest two clades that correspond to separate geographical regions (west and east of the mid-Aegean trench). However, the phylogenetic relationships among Greek populations of *Orthometopon* spp. are different from the presumed pattern on the basis of morphological evidence. The distinct geographical distribution of the major clades of the phylogenetic tree and its topology suggest a spatial and temporal sequence of phylogenetic separations, which coincide with some major palaeogeographical separations during the geological history of the Aegean Sea. The results stress the need for a reconsideration of the evolutionary history of *Orthometopon* species, which will help overcome difficulties encountered in classical taxonomy at the species level.

ADDITIONAL KEYWORDS: Aegean region – evolution – isopods – molecular clock.

INTRODUCTION

The use of molecular data to discern phylogenetic relationships among terrestrial isopod (Crustacea, Isopoda, Oniscidea) species has only recently been undertaken for a few groups of isopods (Marcadé *et al*., 1999; Michel-Salzat & Bouchon, 2000; Mattern & Schlegel, 2001; Rivera *et al*., 2002; Wetzer, 2002; Charfi-Cheikhrouha, 2003; Mattern, 2003; Taiti *et al*., 2003; Klossa-Kilia, Kilias & Sfenthourakis, 2005, 2006). Some previous attempts towards the formulation of phylogenetic hypotheses among oniscideans had been based on anatomical/morphological characters, such as musculature and appendage structure (Schmalfuss, 1989; Wägele, 1989, 1994; Erhard, 1995, 1996, 1997, 1998; Leistikow, 2001; Leistikow & Schmidt, 2002; Schmidt, 2002).

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The genus *Orthometopon* (Verhoeff, 1917) (Agnaridae), according to the definition given by Schmalfuss (1993), is monophyletic and is distributed in south-eastern Europe and western Turkey (Schmalfuss, 2003). Nevertheless, because the differences between named species are generally small and restricted to a few characters, most of which are subject to intraspecific variation, a resolution of the phylogenetic relationships between *Orthometopon* species should begin from a local population basis, and use additional evidence such as molecular markers. A similar approach concerning another terrestrial isopod genus (Klossa-Kilia *et al*., 2005, 2006) has revealed unexpected patterns of relationships that do not always agree with morphological evidence.

As a first step, in the present study *Orthometopon*-spp. specimens were collected from several localities, so that a fairly wide range of populations, presumably also including most species recorded from Greece, are now available for phylogenetic analysis. The DNA

Table 1. List of the specimens of *Orthometopon* examined, with population map codes (see Fig. 1), taxon name, number of samples, geographical origins, and accession numbers. Individuals from two other terrestrial isopod species were used as outgroup taxa: *Ligidium* sp. and *Armadillidium vulgare*

Code	Species	Samples	Locality	Acc. Numbers
1	*O. dalmatinum* (Verhoeff, 1901)	1	Imvros, Crete Island, Greece	EF568937
2	*O. dalmatinum* (Verhoeff, 1901)	1	Elos, Crete Island, Greece	EF568938
3	*O. dalmatinum* (Verhoeff, 1901)	1	Idi Mountain, Crete Island, Greece	EF568939
4	*O. dalmatinum* (Verhoeff, 1901)	1	Kithira Island, Greece	EF568940
5	*O. dalmatinum* (Verhoeff, 1901)	1	Taygetos, Peloponnesos, Greece	EF568941
6	*O. dalmatinum* (Verhoeff, 1901)	1	Arta, Sterea Ellada, Greece	EF568942
7	*O. dalmatinum* (Verhoeff, 1901)	1	Gkamila, Sterea Ellada, Greece	EF568943
8	*O. kerkinianum* ? Schmalfuss, 1993	1	Kerkini, Macedonia, Greece	EF568944
9	*O. phaleronense* (Verhoeff, 1901)	1	Evia, Sterea Ellada, Greece	EF568945
10	*O. phaleronense* (Verhoeff, 1901)	1	Stouronisia, Sterea Ellada, Greece	EF568946
11	*O. phaleronense* (Verhoeff, 1901)	1	Atiki, Sterea Ellada, Greece	EF568947
12	*O. phaleronense* (Verhoeff, 1901)	2	Andros Island, Cyclades, Greece	EF568948
13	*O. phaleronense* (Verhoeff, 1901)	1	Dilos Island, Cyclades, Greece	EF568949
14	*O. phaleronense* (Verhoeff, 1901)	1	Kea Island, Cyclades, Greece	EF568950
15	*O. phaleronense* (Verhoeff, 1901)	1	Folegandros Island, Cyclades, Greece	EF568951
16	*O. phaleronense* (Verhoeff, 1901)	1	Kythnos Island, Cyclades, Greece	EF568952
17	*O. phaleronense* (Verhoeff, 1901)	1	Sifnos Island, Cyclades, Greece	EF568953
18	*O. phaleronense* (Verhoeff, 1901)	1	Kimolos Island, Cyclades, Greece	EF568954
19	*O. phaleronense* (Verhoeff, 1901)	1	Serifos Island, Cyclades, Greece	EF568955
20	*O. turcicum* Verhoeff, 1941	2	Agiasos, Lesvos Island, East Aegean, Greece	EF568956
21	*O. ferrarai* ? (Schmalfuss, 1983)	1	Skala, Lesvos Island, East Aegean, Greece	EF568957
22	*Orthometopon* sp.	1	Samos Island, East Aegean, Greece	EF568958
23	*Orthometopon* sp.	1	Ikaria Island, East Aegean, Greece	EF568959
24	*Orthometopon* sp.	1	Patmos Island, East Aegean, Greece	EF568960
25	*O. scheuerni* Schmalfuss, 1993	3	Mugla, Turkey	EF568961
Outgroup	*Ligidium* Brandt, 1833		–	DQ182804
Outgroup	*Armadillidium vulgare* (Latreille, 1804)		–	AF255779

The question marks indicate specimens with uncertain taxonomy.

sequences were obtained from the cytochrome oxidase I (COI) gene in order to infer the phylogenetic relationships of these species. These were combined with previously published sequences in order to: (1) examine the validity of the current taxonomy; (2) produce a historical interpretation of the distribution of the species; and (3) compare the molecular phylogeny of the genus with that proposed by Schmalfuss (1993) on morphological grounds.

MATERIAL AND METHODS

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

The numbers and the geographical origins of the specimens used in this study are given in Table 1, and are illustrated in Figure 1. All specimens used in the analysis were whole animals stored in 70% ethanol, which is known to create great problems in DNA retrieval (Barnes *et al*., 2000; Schander & Halanych, 2003; Austin & Melville, 2006). For these reasons, specialized techniques for total genomic DNA extraction from museum specimens were used: tissue samples were washed between one and three times in 1 mL of 10 mM Tris-HCl (pH 8.0) on a rotary mixer for 24 h per wash, so as to remove residual ethanol and to rehydrate the tissues (Austin & Melville, 2006). Genomic DNA was then extracted using a DNeasy tissue extraction kit (QIAGEN) following the manufacturer's instructions.

A partial sequence of the mitochondrial protein encoding COI was targeted. Two pairs of primers were used for each DNA extract, following the technique of nested PCR. The first pair of primers

Figure 1. Map showing the sampling localities of the 29 specimens used for the DNA analysis. The numbers correspond to those listed in Table 1.

(LCO, 5′-GGTCAACAAATCATAAAGATATTGG-3′; HCO, 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer *et al*., 1994) specified a 650-bp fragment of the COI gene. These primers amplify homologous sequences in a wide range of animal species, and have been used to generate sequence data for phylogenetic analysis. Thermocycling was performed in a PTC-100 thermocycler (MJ-Research), with an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 47 °C, and 1 min at 72 °C, with a final extension of 72 °C for 10 min.

Then, the primary PCR product was directly used for another amplification reaction, without further purification, with the second pair of primers (C1-J-1718, 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′; C1-J-2191, 5′-CCCGGTAAAATTAAAATATAAACTTC-3′; Simon *et al*., 1994), which amplified a ~470-bp fragment. The conditions for the secondary amplification reaction were the same as those of the primary amplification reaction. All amplification reactions were carried out in a final volume of 20 µL. Each reaction contained 50 mM Tris-HCl pH 8.8, 20 mM NH_4SO_4, 2.5 mM $MgCl_2$, 100 mM of each dNTP, 0.3 U AmpliTaq DNA polymerase (Perkin Elmer), 40 ng of each primer, and approximately 1–20 ng of template DNA. Bovine serum albumin (BSA, 160 µg mL^{-1}) was added to the reactions to help overcome the effect of a powerful PCR inhibitor of unknown origin. A blank reaction containing no DNA was set up as part of an experiment to monitor any possible contamination of PCR reagents.

PCR products were purified with the QIAquick PCR purification kit (Qiagen). Single-stranded sequencing of the PCR product was performed in both directions, using the Big-Dye Terminator (v3.1) Cycle Sequencing kit on an ABI 377 automated sequencer, following the manufacturer's protocol. Primers used in cycle sequencing were those used in PCR amplification. Individuals from two other terrestrial isopod species were used as outgroup taxa: *Ligidium* sp. (DQ182804; Klossa-Kilia *et al*., 2006) and *Armadillidium vulgare* (AF255779; Wetzer,

2001). GenBank accession numbers for the sequences produced for the phylogeny of *Orthometopon* spp. are shown in Table 1.

Alignment and genetic divergence

The alignment of the COI sequences was performed with ClustalX (Thompson *et al*., 1997). The computer-generated alignment was further adjusted manually. Alignment gaps were inserted to resolve length differences between sequences. COI sequences were translated into amino acids prior to analysis, and did not show any stop codons. Sequence divergences were estimated based on Tamura & Nei's (1993) model of evolution in MEGA (v.3.1; Kumar, Tamura & Nei, 2004).

Phylogenetic analyses

Phylogenetic inference analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) methods. Nucleotides were used as discrete, unordered characters.

Maximum parsimony analysis was performed with PAUP* (v.4.0b10; Swofford, 2002), with branch-and-bound searches. Confidence in the nodes was assessed by 1000 bootstrap replicates (Felsenstein, 1985), with the random addition of taxa.

The model used for the ML and BI analyses [General Time Reversible (GTR); Rodriquez *et al*., 1990 + gamma (G)] was selected using Modeltest 3.7 (Posada & Crandall, 1998) under the Akaike's information criterion (AIC; Akaike, 1974). Heuristic ML searches were performed in PAUP* with ten replicates of random sequence addition, and TBR branch swapping using the search strategy of successive approximations (Swofford *et al*., 1996; Sullivan *et al*., 2005).

The analysis of BI was performed in MrBayes (v3.1; Ronquist & Huelsenbeck, 2003), with four runs and four chains for each run for 10^7 generations, and the current tree was saved to file every 100 generations. Full parameter estimation was performed during the tree search. A majority rule consensus tree ('Bayesian' tree) was then produced from the posterior distribution of trees, and the posterior probabilities were calculated as the percentage of samples recovering any particular clade (Huelsenbeck & Ronquist, 2001), where probabilities $\geq 95\%$ indicate significant support. Two further independent Bayesian analyses were run so that global likelihood scores, individual parameter values, topology, and nodal support could be compared to check for local optima.

Molecular-clock calibration and divergence times

A log-likelihood ratio test was used to examine the clock-like evolution of sequences in the combined data set by calculating a χ^2 statistic (likelihood ratio test, LRT), based on ML values both with and without the enforcement of rate constancy [$\chi^2 = -2(\ln L_{\text{CLOCK}} - \ln L_{\text{UNCONSTRAINED}})$, d.f. = number of terminal nodes – 2; Felsenstein, 1981). To estimate divergence times, the net nucleotide divergence (Da) between geographical groups was calculated from Tamura & Nei's (1993) pairwise distances, using the software MEGA. This metric corresponds to the between-group variation corrected for the within-group variation in haplotypes, and can be used to calculate the splitting time of groups (Nei, 1987).

RESULTS

Of the 450 nucleotide sites examined there were 111 (24.6%) variable sites, of which 108 (23.7%) were parsimony informative (151 and 116, respectively, when outgroups were included in the analysis). *Orthometopon* sequence divergence ranged from 0 to 18.4% (Table 2).

Tree-length distribution, determined from random sampling of 10^6 unweighted trees, was significantly skewed to the left (g_1 statistics = –0.572), suggesting a strong phylogenetic signal in the data ($P < 0.01$; Hillis & Huelsenbeck, 1992). Unweighted parsimony analysis of the 161 parsimony-informative characters produced eight equally parsimonius trees with a length of 268 steps (consistency index, CI = 0.769; retention index, RI = 0.922). Maximum likelihood analysis under the GTR + G model resulted in a topology with $\ln L = -1896.16$, which is consistent with the MP analysis (final parameter estimates: base frequencies A = 0.27, C = 0.17, G = 0.18, T = 0.38, shape value (a) = 0.2726, invariable sites (Pinv) = 0, A/C = 1.91, A/G = 4.85, AT = 2.90, CG = 0.77, and C/T = 8.11).

Bayesian inference under the same model of evolution resulted in a topology with mean $\ln L = -1905.614$. Identical topologies were recovered for each of the four runs with the full dataset, and the 50% majority-rule consensus tree of the 99×10^3 trees remaining after burn-in are presented in Figure 2.

In all phylogenetic analyses two well-supported clades were identified, corresponding to different groups of species and/or to separate geographical regions throughout the Aegean region. Clade A (the eastern clade), which branched off first, comprised *Orthometopon* specimens from eastern Aegean islands and from Turkey (MP bootstrap value/ML bootstrap value/BI posterior probability = 95/92/0.99, respectively). This could be further subdivided into two lineages (A1 and A2), which are in accordance with the geographical origin of the specimens: subclade A1 (98/99/0.99) includes populations from Turkey (*Orthometopon scheuerni*), whereas subclade A2 (62/64/

Table 2. Nucleotide divergences (%, Tamura–Nei model) of cytochrome oxidase I (COI) sequences among the major mitochondrial DNA clades/lineages of *Orthometopon* included in the study. The net nucleotide divergences (*Da*) are given within parentheses

	1	2	3	4	5	6	7
Subclade B1.2 (Pel-Kyt-Gr)	–						
Subclade B1.1 (Crete)	9.4 (8.6)	–					
Subclade B2.1 (Kyc-Att)	10.6 (10.0)	14.2 (13.9)	–				
Subclade B2.2 (Kerkini)	10.1 (10.1)	12.8 (12.8)	4.8	–			
Subclade A2.1 (Lesvos)	14.1 (13.7)	15.4 (14.9)	14.8 (14.4)	14.1 (14.1)	–		
Subclade A2.2 (East Aegean)	14.4 (13.9)	15.7 (15.3)	15.1 (14.7)	14.2 (14.2)	1.1 (1.1)	–	
Subclade A1 (Turkey)	14.6 (14.1)	18.4 (17.8)	14.8 (14.3)	13.8 (13.8)	2.6 (2.5)	3.1 (3.0)	–
Outgroup	24.5	26.4	23.7	24.4	22.0	23.1	22.7

The definitions of subclades were based on the phylogentic tree of Figure 2.
Abbreviations: Att, Attiki; Gr, Continental Greece; Kyc, Kyklades; Kyt, Kythira; Pel, Peloponnesos.

0.94) includes populations from the eastern Aegean islands (*Orthometopon turcicun*, and possibly *Orthometopon ferrarai*).

Clade B (the western clade) consists of *Orthometopon* specimens from several continental Greek localities: the Kyklades island group, and the islands of Kythira and Crete (94/88/0.97), and could be further subdivided into two subclades. Subclade B1 includes populations of *Orthometopon dalmatinum* from continental Greece and Crete (89/85/0.96), which was also divided into two well-supported subgroups of haplotypes that correspond to two geographically distinct clusters of areas. The first consisted of the island of Crete (100/100/1.00), and the second consited of the continental Greek localities (Peloponnisos, central Greece) and the island of Kythira (90/83/0.95). Subclade B2 includes specimens from continental Greece and the Kyklades island group (99/98/1.00), and could be further subdivided into two well-supported monophyletic groups. The first contains the specimen from Kerkini (*Orthometopon kerkinianum*?), whereas the other comprises specimens of the same species (*Orthometopon phaleronense*) from several Kyklades islands, Evvoia, and Attiki (91/88/0.98). However, the relationships within this subgroup are considered unresolved because of the low bootstrap support in all the phylogenetic analyses.

The likelihood-ratio test did not reject the null hypothesis of a homogeneous clock-like rate of evolution for the tree produced by the *Orthometopon* sequences from Greece [LRT = 2(1418.60 – 1436.49) = 35.78, d.f. = 27, $\chi^2_{0.05} = 40.11$]. This result suggests that we can use the genetic distances between populations inhabiting different geographical regions, in conjunction with the geological information about the age of the tectonic events that are responsible for the separation of these regions, in order to estimate a local rate of evolution for the *Orthometopon* species.

DISCUSSION

The phylogenetic relationships among Greek populations of the terrestrial isopod genus *Orthometopon*, based on COI, are different than the presumed pattern suggested by Schmalfuss (1993) on the basis of morphological evidence. More precisely, Schmalfuss (1993) had suggested a scheme where *O. phaleronense* is the sister species of *O. turcicum*, with *Orthometopon hydrense* and *O. kerkinianum* belonging to the same clade, but with unresolved relationships within the clade. Also, *O. dalmatinum* belongs to a different clade, together with the central European species *Orthometopon planum* and with *O. scheuerni*. Finally, *O. ferrarai* is placed at a basal clade, as the sister group of all other species.

Even though we did not use specimens of *O. planum*, *O. hydrense*, and nominal *O. ferrarai* for the present analysis, the relationships among the populations found contradict this scheme in the following points: *O. phaleronense* (populations from the Kyklades islands, Attiki, and Evvoia) with *O. kerkinianum*? (population from Kerkini) form a clade that is the sister clade of *O. dalmatinum* (populations from Crete, Kythira, and western continental Greece), whereas *O. turcicum* (population from Lesvos, and possibly also from other eastern Aegean islands – see below) forms a separate clade together with *O. scheuerni* (population from Mugla, Turkey), which appears to be its sister species. Another important finding is that the specimens from the eastern Aegean islands of Samos, Ikaria, and Patmos (subclade A2.2) appear as either *O. turcicum* (subclade A2.1), or at least as a species very closely related to it. Moreover, the genetic distances among the specimens of clade A, especially between the subclades A2.1 and A2.2, were very low (1.1–3.1%, see Table 2). These values are consistent with specimens belonging to the same

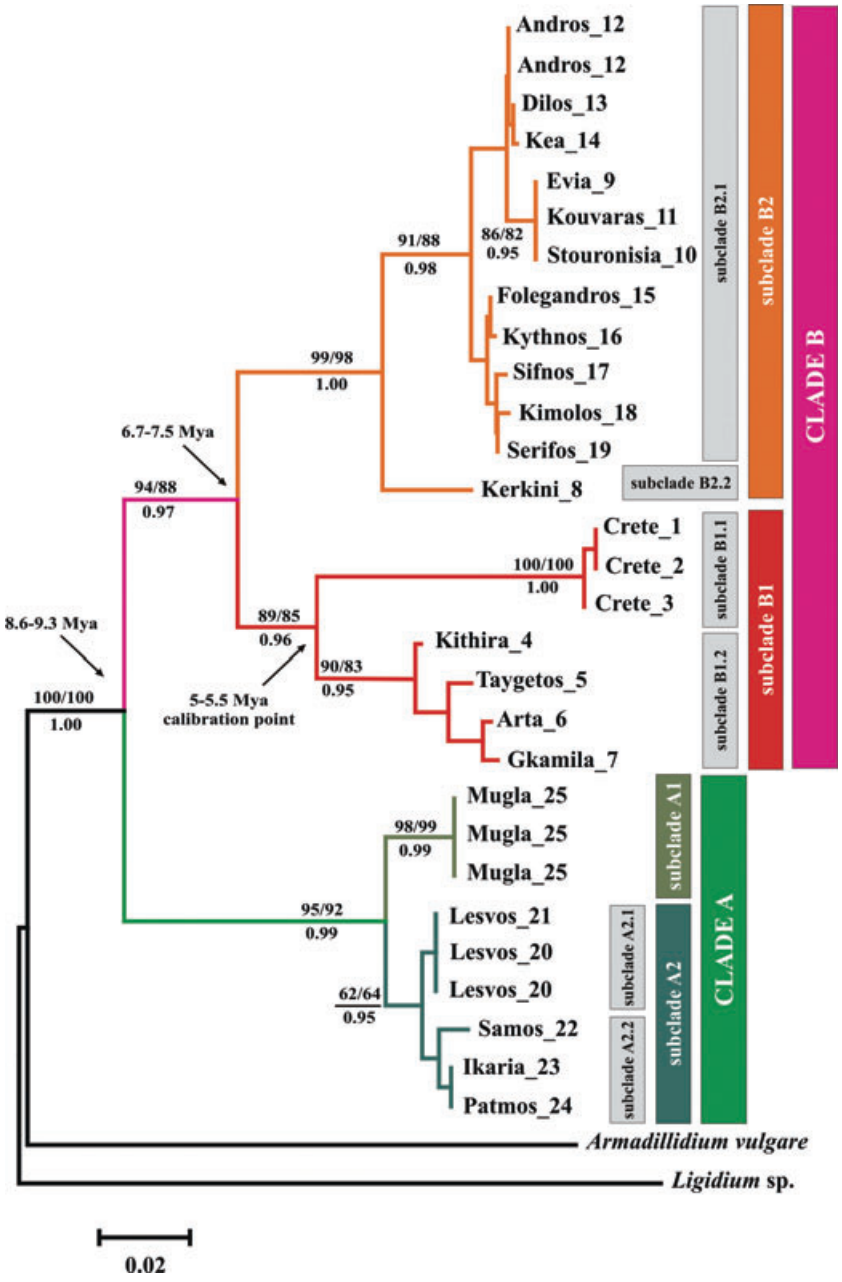

Figure 2. Phylogenetic relationships among the 29 specimens of *Orthometopon* species. Individuals from two other terrestrial isopod species were used as outgroup taxa: *Ligidium* sp. and *Armadillidium vulgare*. Phylogenetic analyses, maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI), all produced trees with the same topology. Only the BI tree is presented here. Numbers above the branches indicate bootstrap values in the MP and ML analyses, respectively (MP/ML). Numbers below the branches indicate the posterior probabilities of the Bayesian analysis (BI).

species, and are comparable with the mean nucleotide divergence of the other subclades of this tree (i.e. 1.1% for subclade B2.1), indicating that the taxonomy of this clade (A) requires revision. According to the literature (Sfenthourakis, 1996), the species recorded from these islands (Samos, Ikaria, and Patmos) is *O. phaleronense* (identified from morphological evidence). The results of our study show that this was a misidentification, probably arising from the variability of the traditional diagnostic characters. We used the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999) to test whether the cladograms predicted

by alternative phylogenetic hypotheses (monophyly of *O. phaleronense*) were significantly different from the tree obtained in our analyses. This test rejects the hypothesis that *O. phaleronense* (including the specimens from Samos, Ikaria, and Patmos) is monophyletic ($P < 0.001$), providing further support of this assertion (implemented in PAUP*, RELL bootstrap with 1000 replicates, $P < 0.001$). These populations should be tentatively included in *O. turcicum*, until a more detailed revision of the genus becomes available. Such an interpretation is also more reasonable from a biogeographical point of view, as these islands were part of the Asia Minor mainland until a few thousand years ago, but were permanently separated from the Kyklades in the Miocene (Dermitzakis & Papanikolaou, 1981).

The present phylogenetic pattern, if confirmed by further studies, indicates that the morphological characters, which were used in the diagnoses of species for phylogenetic inference, are not valid. Schmalfuss (1993), for example, considers the presence of a transverse band ('Querleiste') in pereon-tergites II–VI as a synapomorphy of the *planum-dalmatinum-scheuerni* clade. Given that we lack information regarding the development and functional ecology of these organisms, as well as a robust phylogeny at a broader taxonomical level (e.g. of genera within the family Agnaridae), any interpretation of such a character is arbitrary. It could easily be a convergence or even a symplesiomorphy. The same can be said for the presumed synapomorphy of *O. phaleronense* and *O. turcicum*: i.e. the form of wax-rings ('Wachskringel') on tergite surfaces. The function and formation of these structures are not known, so we cannot base inferences for phylogeny on their presence or absence.

Another seemingly important character, the presence of pleopodal lungs on pleopod-exopodites IV and V, supposed to have evolved separately in different clades (Schmalfuss, 1993), is now better resolved on the cladogram. Of the nominal species used in the present study, this character is present in *O. ferarrai* (one specimen from Lesvos island – sublade A2.1) and *O. scheuerni* (from Mugla, Turkey – subclade A1). One polymorphism (on Lesvos island) and one loss (in subclade A2.2) are now required for this character. Alternatively, we could assume that this character is present in all members of clade A, with a misidentification of its state in the specimens from the eastern Aegean islands. In such a case, this character retains its important status as a synapomorphy of clade B. Further detailed microscopic study of the pleopods from these populations is required before a final assessment on its relevance for *Orthometopon* phylonegy reconstruction is needed.

The LRT shows that the molecular-clock hypothesis cannot be rejected. This indicates a homogeneous clock-like rate of evolution for the tree produced by the *Orthometopon* sequences included in this study. Consequently, it is possible to calibrate a 'local clock' for *Orthometopon*, using at least one independently timed palaeogeographical event (calibration point). From geological events, the isolation of the island of Crete is well dated (some 5.5–5 Mya; Meulenkamp, 1985), and corresponds to the divergence of the subclade B1.1 (populations of Crete) from subclade B1.2 (Kythira, Peloponnesos, and continental Greece). Seeing that the corrected net pair-wise divergence (*Da*) between subclade B1.1 and subclade B2.2 is 8.6%, the evolutionary rate is calculated to be 1.56–1.72% per million years. On the basis of these evolutionary rates we infer that the diversification of *Orthometopon* occurred 8.5–9.3 Mya (the *Da* between clade A and clade B is 14.6%), during the late Miocene (Fig. 2), whereas the separation of subclade B1 from subclade B2 occurred 6.8–7.5 Mya (the *Da* between B1 and B2 is 11.7%).

From a phylogeographical point of view, the phylogenetic pattern revealed by COI sequences is fairly reasonable. The separation into two clades, an eastern clade (clade A) and a western clade (clade B) containing continental Greece and the central Aegean islands, is in accordance with established palaeogeography of the region. The forming of a Mid-Aegean trench (east of Crete and west of Kasos–Karpathos) began at the end of the middle Miocene (12 Mya) and was fully completed during the early late Miocene (10–9 Mya) (Creutzburg, 1963; Dermitzakis & Papanikolaou, 1981), causing the separation of the western Aegean from the eastern Aegean islands. Several other taxa, both of isopods (Klossa-Kilia *et al*., 2006) and other animals (e.g. Poulakakis *et al*., 2003; Parmakelis *et al*., 2006), exhibit a similar pattern. Subclade B1 also shows a reasonable sequence of divergences, with Crete diverging first and forming a well-supported and differentiated clade, followed by the island of Kythira, and finally by the continental populations. Indeed, Crete has been separated from continental Greece for at least 5 Myr (Dermitzakis & Papanikolaou, 1981; Schüle, 1993), whereas Kythira was part of southern Peloponnisos in the Pleistocene (Schüle, 1993). Unfortunately, we do not have COI sequences of *O. planum*, so the position of this widely distributed European species cannot be evaluated, but on biogeographical grounds it should be the sister species of *O. dalmatinum*. The inclusion of this species in future analyses may resolve the exact status of Cretan populations that, according to their divergence, could be regarded a separate new species. If *O. planum* is placed between the Cretan and continental Greek populations, such an interpretation will be unequivocally substantiated. At present, we can only suggest the consideration of Cretan populations as a new endemic taxon.

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