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Molecular phylogenetic relationships among some stygobitic cirolanid species (Crustacea, Isopoda)

Mariella Baratti[1], Mariateresa Filippelli[1], Francesco Nardi[2], Giuseppe Messana[1]
[1] *Institute for the Study of Ecosystems, CNR, Via Madonna del Piano 10, Sesto Fiorentino (FI) 50019-I, Italy*
[2] *Department of Evolutionary Biology, University of Siena, Via A. Moro 2, Siena, 53100-I, Italy*
[3] *E-mail: baratti@ise.cnr.it*

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Abstract

Within the Cirolanidae, a widespread family of marine isopods, about 23 genera are stygobitic and inhabit phreatic and anchialine ecosystems, with many endemic species. The Mediterranean area has a high biodiversity of subterranean cirolanids, which are considered thalassoid limnostygobionts. A molecular analysis was conducted using mtDNA genes to infer the phylogeny of species belonging to six of the seven stygobitic genera of Cirolanidae inhabiting the Mediterranean basin and to two American taxa: *Faucheria faucheri*, *Marocolana delamarei*, *Saharolana seurati*, *Sphaeromides virei virei*, *Turcolana* sp., 13 taxa of the genus *Typhlocirolana* and two American species, *Antrolana lira* and *Speocirolana bolivari*. The *Typhlocirolana* species are widespread in the western Mediterranean basin, with a concentration of taxa in the Maghreb region. *Turcolana* sp. is localised in the eastern Mediterranean, while *F. faucheri* and *S. v. virei* are north Mediterranean taxa. *S. seurati*, the taxon least morphologically adapted to subterranean life, belongs to a monospecific genus present in a Tunisian spring. The molecular phylogeny showed a high affinity among the American taxa and the Mediterranean *Sphaeromides*, clustering in the *Sphaeromides* group identified by previous morphological studies. *Typhlocirolana* species and *M. delamarei* constitute their sister clade within the *Sphaeromides* group. *F. faucheri* appears to be a sister clade of the *Sphaeromides* group. *S. seurati*, showing reduced troglobitic adaptations, assumes disparate and unsolved positions in the phylogenetic reconstructions. The molecular data suggest that a combination of vicariance and dispersal events, occurring from 180 to a few million years ago, combined to bring about the present distribution pattern of Mediterranean cirolanid isopods.

Contents

Introduction

Despite the importance of subterranean waters, the biodiversity of these ecosystems is still poorly investigated from a genetic point of view, even though there has been an increase in the number of studies in recent years (Ketmaier *et al*., 2003; Baratti *et al*., 2004; Verovnik *et al*., 2004, 2005; Lefebure *et al*., 2006; Buhay *et al*., 2007; Finston *et al*., 2007; Porter *et al*., 2007; Zakšek *et al*., 2007; Foulquier *et al*., 2008; Page *et al*., 2008; Carlini *et al*., 2009). Molecular studies of the subterranean fauna have produced interesting results and new directions in the field of biospeleology. For instance, molecular analyses of groundwater fauna have led to possible explanations of its origin (Ketmaier *et al*., 2003; Baratti *et al*., 2004; Zakšek *et al*., 2007). Groundwater aquifers are controlled by geological and hydrological processes, historical changes and seasonal patterns, which influence the distribution patterns of stygobionts inhabiting them (Finston *et al*., 2007). Several hypotheses have been proposed regarding the colonization of subterranean waters by faunas coming from nearby superficial biotopes (marine or freshwater) and probably subject to bottlenecks and/or long stable isolation typical of these ecosystems (Holsinger, 2000; Trajano, 2005; Buhay *et al*., 2007). In particular, the so-called thalassoid limnostygobionts present distribution patterns that are traditionally explained by marine transgression and regression cycles starting from marine benthic ancestors that invaded littoral habitats (Humphreys, 2000).

The Cirolanidae, a predominant marine family with about 300 known species, is one of the two families of Isopoda Flabellifera that include 23 stygobitic genera (the other family being Sphaeromatidae) (Botosaneanu, 1986). In the Mediterranean area, the subterranean genera mainly belong to the subfamily Cirolaninae (sensu Hansen, 1905; Botosaneanu, 1986; Wägele,

1989; Herrando-Perez *et al*., 2007). Within this subfamily, three groups were identified on the basis of morphological characters (Botosaneanu, 1986): the *Cirolana* group, comprising (as stygophilic elements) the monospecific microphthalmic, crenobitic *Saharolana* Monod, 1930 and the non-Mediterranean *Anopsilana* Paulian and Delamare Deboutteville, 1956, *Creaseriella* Riojia, 1953 and *Haptolana* Bowman, 1966; the *Sphaeromides* group, comprising *Sphaeromides* Dollfus, 1897, *Turcolana* Argano and Pesce, 1980, *Typhlocirolana* Racovitza, 1905 and the American *Antrolana* Bowman, 1964, *Bahalana* Carpenter, 1981, *Cirolanides* Benedict, 1896, *Mexilana* Bowman, 1975, *Oncilorpheus* Paul and Menzies, 1971 and *Speocirolana* Bolivar and Pieltain, 1950; the *Faucheria* group, comprising *Faucheria* Dollfus and Vire, 1905, the African *Skotobaena* Ferrara and Monod, 1972, the American *Sphaerolana* Cole and Minckley, 1970 and the recently described *Kensleylana* Bruce and Herrando-Perez, 2005 from Spain. In the Mediterranean area there are seven genera of Isopoda: *Faucheria* and *Kensleylana* (*Faucheria* group), *Saharolana* (*Cirolana* group) and *Typhlocirolana*, *Marocolana*, *Sphaeromides*, *Turcolana* (*Sphaeromides* group). The species belonging to these genera show an evident troglomorphic aspect, with the exception of the taxon *Saharolana seurati* characterized by stygophilic characters, such as microphtalmy.

Stygobitic cirolanid isopods are characterized by high biodiversity in the Mediterranean area and they represent a very interesting group for research on evolutionary trends in subterranean ecosystems, acting as good palaeogeographic indicators because of their poor dispersal abilities (Boutin and Coineau, 2000). Cirolanid stygobitic isopods frequently occur in areas that were covered by the Tethys Sea and they are considered Tethyan relicts, like other crustacean taxa (Fakher El Abiari *et al*., 1999; Jaume and Christenson, 2001; Zakšek *et al*., 2007). These stygobionts consist of species considered representatives of the thalassoid limnostygobitic fauna (*i.e*. derived from coastal marine populations, Boutin and Coineau, 2000), whose direct marine ancestors are unknown.

The importance of applying molecular methods to the study of Cirolanidae is related to crypticism, morphological convergences and the strength and significance of diagnostic characters used in the phylogeny of cirolanid stygobitic isopods (Monod, 1930; Nourisson 1956; Margalef, 1958; Wägele, 1989; Iliffe and Botosaneanu, 2006), all of which make the systematics of the group very difficult. The morphological adaptations to hypogean life and the high endemisms of the stygobitic species mean that molecular investigations of the group are essential to clarify the phylogenetic relationships and molecular evolutionary trends. Previous molecular studies mainly carried out on the genus *Typhlocirolana* Racovitza 1905 (Baratti *et al*., 1999, 2004) suggested a primary evolutionary role of vicariance in the evolution of Cirolanidae, driven by the palaeogeographic events occurring in the western Mediterranean (Rosenbaum *et al*., 2002). In the present study, we analysed this group of taxa using the two gene portions, 12S and 16S, already utilized in previous works (Baratti *et al*., 1999, 2004). The usefulness of these molecular markers to infer phylogeny in Crustacea at various taxonomic levels has been well established (Taylor *et al*., 1996; Kitaura *et al*., 1998; Salzat and Bouchon, 2000; Schubart *et al*., 2006). Moreover,

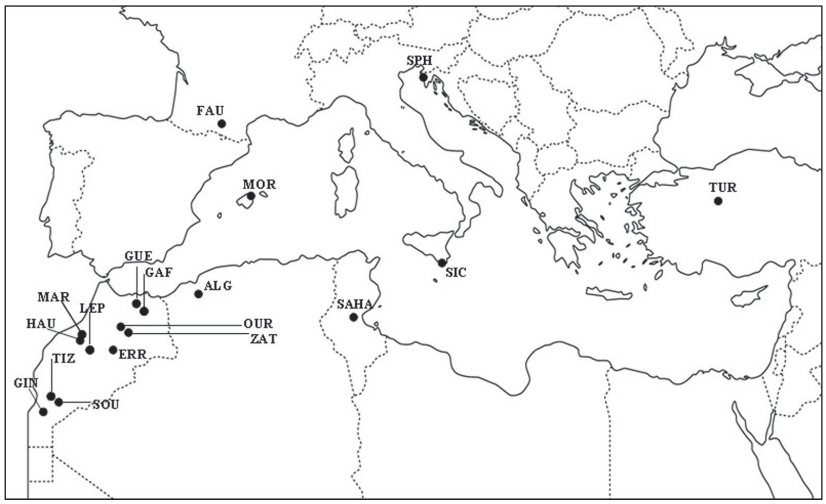

Fig. 1. Distribution of the analysed Mediterranean taxa, *Antrolana lira* and *Speocirolana bolivari* from America are excluded. Abbreviations as in Table 1.

Table 1. Species used in this analysis. The new data set is underlined; the other is from a previous study (Baratti *et al*., 2004). Asterisks indicate taxa from other authors (see GenBank accession number).

sample population	abbreviation	locality	GenBank accession number		
			16S gene	12S gene	COXI
Antrolana lira Bowman, 1964	ANTRO	Virginia (USA)	EF460862	EF460861	
**Cirolana rugicauda* Heller, 1861	CIR	South of Luderitz (Namibia)	AF259544	AF259530	AF259530
Faucheria faucheri (Dollfus and Virè, 1900)	FAU	Avencas, Montpellier (France)	DQ373040	DQ373039	
Marocolana delamarei Boutin, 1993	MAR	Marrakech (Morocco)	DQ194363	AF356858	EF526114
Saharolana seurati Monod, 1930	SAHA	Chott el Jerid (Tunisia)	DQ194359	DQ194368	
Speocirolana bolivari (Rioja, 1953)	SPEOC	Tamaulipas (Mexico)	EF460864	EF460863	
Sphaeromides virei virei (Brian, 1923)	SPH	Labin (Croatia)	DQ194360	DQ194369	AY998015
S. virei mediodalmatina Sket, 1964		Žegar Dalmatinski (Croatia)			AY998014
Typhlocirolana haouzensis Boutin, Boulanouar, Coineau and Messouli, 2002	HAU	Marrakech (Morocco)	AF356847	AF356855	EF526113
T. moraguesi Racovitza, 1905	MOR	Balearic Is. (Spain)	AF356849	AF356857	
T. leptura Botosaneanu, Boutin and Henry, 1985	LEP	Marrakech (Morocco)	DQ194365	DQ194367	
T. cf. *fontis*	ALG	Mont Tlemcen (Algeria)	FJ460466	FJ460467	
T. sp. 1	OUR	River Ourika (Morocco)	AF356850	AF356854	
T. sp. 2	ZAT	River Zat (Morocco)	AF356852	AF356853	
T. sp. 3	SOU	Souss (Morocco)	DQ194362	AF356868	
T. sp. 4	TIZ	River Tiznit (Morocco)	DQ194364	AF356864	
T. sp. 5	GIN	Agadir Izder (Morocco)	DQ194361	AF356866	
T. sp. 6	GAF	El Gafayt (Morocco)	AY093577	AY093574	
T. sp. 7	GUE	Guercif (Morocco)	AY093578	AY093575	
T. sp. 8	SIC	Sicily Island (Italy)	AF356851	AF356856	
T. sp. 9	ERR	Errachidia (Morocco)	DQ379984	DQ194366	
Turcolana sp.	TUR	Eskisehir (Turkey)	no data	AJ388073	

to more accurately determine the genetic divergence between *Typhlocirolana haouzensis* Boutin, Boulanouar, Coineau and Messouli, 2002 and *Marocolana delamarei* Boutin, 1993, which showed a very close relationship in the 12S and 16S analysis, we also sequenced a portion of cytochrome oxidase I (COXI). This gene is considered the most reliable marker to infer interspecific genetic distance (Lefebure, 2006), even though many precautions must be adopted to avoid amplifications of COXI-like sequences (Buhay, 2009).

Material and methods

The data set for this study is reported in Table 1. The sampling localities of the studied species in the Mediterranean basin are reported in Fig. 1. Genomic DNA was extracted from entire specimens preserved in absolute ethanol using the Qiamp Tissue kit (Qiagen Inc., USA), following the manufacturer's protocol.

mtDNA sequencing

PCR amplification products were obtained from the 12S and 16S mitochondrial gene portions for all the taxa included in the analysis, with a few exceptions (Table 1). For 12S, we used the primers 12Sbi (5'–AAGAGCGACGGGCGATGTGT–3') (Simon *et al*., 1994) and 12SL4 (5'–GTGCCAGCMGCCGCGGTTA–3') (Schubart *et al*., 2006). Primers used to amplify the 16S gene portion were 16Sar (5'–CGCCTGTTTATCAAAAACAT–3') and 16Sbr (5'–CCGGTCTGAACTCAGATCACACGT–3') (Palumbi *et al*., 1991). The taxa *T. haouzensis*, *M. delamarei* and *S. virei* were also analysed with a portion of Cytochrome Oxidase I using the primers mtd10 universal primer 5' –TTGATTTTTTGGTCATCCAGAAGT -3' (Roehrdanz, 1993) and Florence 5'- CCTAAAAAATGTTGAGGGAA-3' (Baratti *et al*., 2005). The PCR profiles for 12S/16S rRNA amplifications were described by Baratti *et al*. (2004) and for COXI by Baratti *et al*. (2005). PCR products were run on a 1.5% agarose gel,

containing 0.5 g/ml ethidium bromide. The amplification patterns were analysed with the Gel Analysis Program v. 2.0 (Ultraviolet Products Ltd.). PCR products were purified (ExoSAP-IT, Amersham Biosciences), sequenced with a Perkin-Elmer sequencing kit (ABI Big Dye Terminator Cycle Sequencing v. 2.0-ABI PRISM, Applied Biosystems, Foster City, USA) and analysed with an ABI 310 automated sequencer (Applied Biosystems). All the sequences are deposited in GenBank with the accession numbers reported in Table 1.

Sequence analysis and nucleotide diversity

Approximately 480 bp of the 12S rRNA gene and 575 bp of the 16S rRNA gene were sequenced. Unreliable sequences were obtained for the 16S gene portion in six individuals of the taxon *Turcolana* sp., which was not included in the combined data set tree. For COXI, a portion of 432 nucleotides was amplified. All regions were sequenced in both directions. Electrophenograms were visualised with CHROMAS 1.45 (http://www.technelysium.com.au). The sequences were manually corrected and analysed with ProSeq 2.9 Beta (http://helios.bto.ed.ac.uk/evolgen/filatov/proseq.html) and then aligned using ClustalX 1.81 (Thompson *et al*., 1997). Multiple alignments were obtained with ClustalX (Jeanmougin *et al*., 1998) by assigning different gap penalty values (opening: 5, 15, 25; extension: 0.2, 5, 10). The resulting alignments were used to construct tree topologies with the different combinations of gap values. Polymorphic sites, nucleotide statistics and genetic distances (K2P) were analysed with MEGA 2.1 (Kumar *et al*., 2001).

Phylogenetic analysis

A chi-square test of homogeneity of base frequencies across taxa was carried out using PAUP 4.0, b. 10 (Swofford, 2001). We executed the likelihood mapping method (Strimmer and von Haeseler, 1997) using TREE-PUZZLE (Schmidt *et al*., 2002) to test the a priori phylogenetic signal in the mtDNA portions studied. Phylogenetic congruence among 12S and 16S data partitions was also performed using the partition-homogeneity test implemented in PAUP* (Swofford, 2001).

Testing of the evolutionary model that best fits our data was conducted with MODELTEST 3.04 (Posada and Crandall, 1998), based on a likelihood ratio test. Different models of nucleotide substitutions were fitted to each data set and for the combined data set. For 12S, the TRN model was the best one selected (Tamura and Nei, 1993), while for 16S the GTR model was selected (Tavaré, 1986). Both models were corrected for rate heterogeneity among sites with a Gamma (G) distribution (Yang, 1993). For the 12S and 16S data sets, the GTR+G model was selected. We carried out a phylogenetic reconstruction by Maximum Parsimony (MP) (Kluge and Farris, 1969) and Neighbour-Joining (NJ) (Saitou and Nei, 1987) analyses using PAUP and by the Bayesian method using MrBayes 3.0B4 (Huelsenbeck and Ronquist, 2001). MP and NJ analyses were performed separately for each set of DNA sequences and in a combined analysis (total evidence approach) for the taxa sequenced for both genes. Neighbour-Joining trees were constructed with distances computed with the best-fit model obtained with MODELTEST. Parsimony analysis was carried out using the heuristic search algorithm, using 100 random-taxon-replicates for all analyses. The analysis was performed with ACCTRAN optimization and tree bisection TBR branch swapping, considering all characters as unordered and equally weighted, and gaps treated as fifth state. A strict consensus tree was calculated when there was more than one tree. Branch supports were assessed by 1000 non-parametric bootstrap replicates. Non-parametric bootstrapping with heuristic searches of 2000 replicates for MP and NJ was used to assess confidences of branches in MP and NJ. A Bayesian analysis was also performed with MrBayes 3.0B4, with clade support assessed by posterior probability. Four Markov chains, one heated and three cold, were allowed to run for two million generations using random starting trees, trees were sampled every 100 generations, with a burnin amounted to 20%.

Regarding the choice of outgroups for the Mediterranean stygobitic cirolanids, no marine cirolanid isopods were available to us. However, the direct marine ancestors of stygobitic cirolanids are unknown at present. The marine cirolanids described for the Mediterranean sea are 12 species belonging to three genera (*Eurydice, Cirolana, Natatolana,* Bruce, 1986; Keable, 2006). No samples of these taxa were collected during our research activities and we used as outgroup a marine cirolanid taxon present in the GenBank Database: *Cirolana rugicauda* Heller, 1861.

Divergence time estimates

A likelihood-ratio test (LRT) was performed to test the molecular clock hypothesis based on 16S gene sequences. The likelihoods with the molecular clock

assumption (L0) and without the molecular clock assumption (L1) were calculated using PHYLIP (Phylogeny Inference Package 3.66, Felsenstein, 2005). The molecular clock hypothesis was never rejected between all the *Typhlocirolana* species and *Marocolana*, while it was always rejected in comparisons involving *Sphaeromides*, *Antrolana*, *Speocirolana*, *Faucheria* and *Saharolana*.

Since the clock-like behaviour of the data was rejected for the complete dataset, a relaxed clock (Uncorrelated Lognormal) was applied, as implemented in BEAST 1.5.3 and accompanying utilities (Drummond and Rambaut, 2007) using a Bayesian MCMC approach. The model of sequence evolution was set according to MODELTEST, and a Yule tree prior was applied. In the absence of suitable fossil calibration points, a strong prior was set for the rate of sequence evolution, applying a mean rate of 0.00325 changes per site per myr. This corresponds to a pair-wise divergence rate of 0.65% per myr, proposed for the 16S gene of crustaceans, in particular of isopods, by Held (2001). Following preliminary runs, the final analysis consisted in two independent runs of 10 million generations each, logging trees and parameters every 1000 generations. Effective sample size (ESS) values, parameter traces and frequency plots were examined with Tracer 1.5 (Rambaut and Drummond, 2003) to confirm stability as well as consistency between the two runs. The initial 10% of generations were removed from the analysis as burnin. Log and trees files were combined with LogCombiner and the posterior distribution of node presence and node height were summarized with TreeAnnotator over the maximum clade credibility tree.

Results

After alignment, 380 base pairs were obtained for the 12S gene: 63 invariable, 187 variable uninformative and 130 parsimony informative. The percentage of genetic divergence at the species level ranges from 6% (*T*. sp. from Zat River vs *T*. sp. from Ourika River) to 36% (*S. v. virei* vs *Saharolana seurati* Monod, 1930). For the 16S gene, we examined 444 aligned base pairs: 99 invariable, 200 variable and 145 parsimony informative. For this gene, the divergence between species ranges from 7% (*T. haouzensis* vs *M. delamarei*) to 42% (*S. seurati* vs *Antrolana lira* Bowman, 1964).

All the sequences are A-T rich (Table 1), in agreement with the observation that arthropod mitochondrial genomes generally tend to be highly A+T biased, even though the A-T content is lower than in other arthropods such as insects (Simon *et al*., 1994; Muraji and Nakahara, 2001). The results of the chi-square homogeneity test show homogeneous base composition within the ingroup taxa for the 12S ($\chi2$= 125.65, $p<0.0001$) and 16S ($\chi2$= 125.80, $p<0.0001$) rRNAs.

The results of the likelihood mapping method carried out with TREE-PUZZLE suggest the presence of a strong phylogenetic signal in the two mitochondrial gene portions. Sequence congruence between the 16S and 12S gene fragments is not rejected according to the Homogeneity Partition test (p=0.45) for those taxa sequenced for both genes. The heuristic search produced two most parsimonious trees at 1878 steps (CI=0.58, RI=0.54, HI=0.41), from which a strict consensus tree is presented (Fig. 2).

The *Sphaeromides* group (SPH-G, sensu Botosaneanu, Table 2) appears to be conserved in all trees (Figs 2-3). It includes the species belonging to the genera *Typhlocirolana*, *Marocolana*, *Antrolana*, *Speocirolana* and *Sphaeromides*, as previously suggested on the basis of morphological investigations. The only exception is *Turcolana* sp., which appears in the 12S tree with an unsolved position and very divergent from all the other Mediterranean stygobitic cirolanid species analysed (Fig. 3). However, the divergence of the 12S sequence in *Turcolana* sp. could also be due to pseudogene amplification (Song *et al*., 2008). 16S amplification performed on six individuals of *Turcolana* sp. yielded unsuccessful products and requires other samples and analyses.

The *Sphaeromides* group presents two major lineages: the clade containing the *Typhlocirolana* species and *M. delamarei*, and the clade containing *S. virei* and the two American taxa, *A. lira* and *S. bolivari* (Rioja, 1953). Within the *Typhlocirolana* group, *T. leptura* Botosaneanu, Boutin and Henry, 1985 and *Typhlocirolana* sp. from Errachidia (ERR) (central-eastern Morocco) show high affinity and are grouped with the taxa (*T.* spp., GIN, TIZ, SOU, OUR, ZAT) inhabiting the central and southern part of Morocco (Fig. 2). On the other hand, *T.* cf. *fontis* from Algeria (ALG) seems to be closely related to the species living in the northern part of Morocco (GAF, GAY), which together with MOR and SIC constitute the M group (a group of species present along or near to Mediterranean coasts), well supported in all trees (Figs 2-3).

The 12S tree confirms the groups detected by the 16S and 12S+16S phylogenies: *S. virei* and the two American taxa; GUE, GAF, ALG, SIC and MOR;

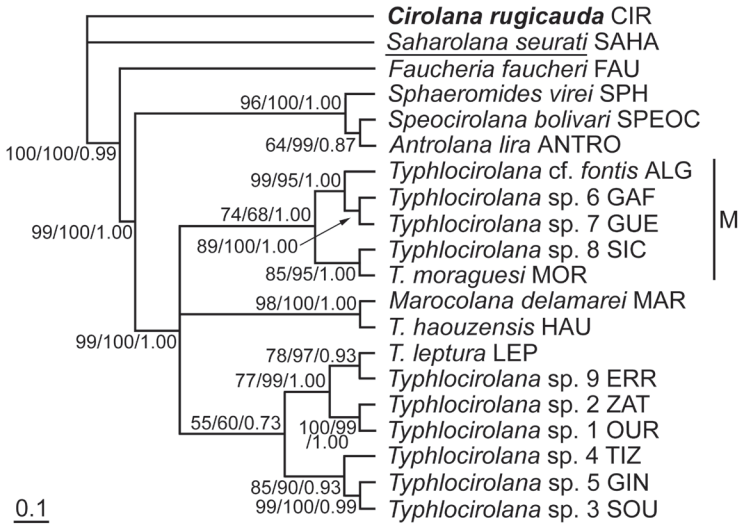

Fig. 2. Consensus of proposed phylogenetic relationships obtained with the combined 16S and 12S data. The consensus diagrams summarize the results of the three reconstruction methods, with their support values for single nodes, Bayesian posterior probability, MP and NJ bootstrap values at the nodes. M: Mediterranean group; SPH-G: *Sphaeromides* group, explanations in the text. Marine species in bold; taxa with light troglomorphology underlined; taxa with troglomorphic characters only in italic. Abbreviations as in Table 1.

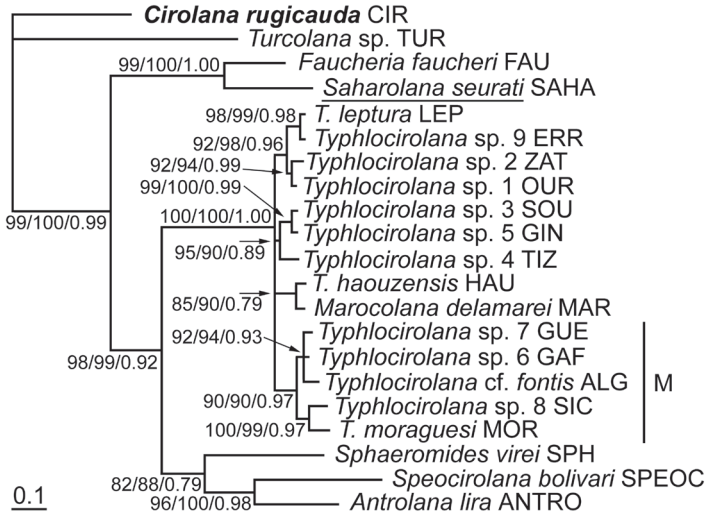

Fig. 3. Bayesian phylogenetic analysis obtained with 12S data. The consensus diagrams summarize the results of the three reconstruction methods, with their support values for single nodes, Bayesian posterior probability, MP and NJ bootstrap values at the nodes. M: Mediterranean group; SPH-G: *Sphaeromides* group, explanations in the text. Marine species in bold; taxa with light troglomorphology underlined; taxa with troglomorphic characters only in italic. Abbreviations as in Table 1.

HAU AND MAR (Fig. 3). In contrast, some differences were detected when the 12S and 16S sequence data were analysed separately: *S. seurati* and *F. faucheri* cluster together as the sister clade of the *Sphaeromides* group (SPH-G), as expected on the basis of the previous studies (Botosaneanu, 1986, Waegele, 1989). In the 12S tree (Fig. 3) the *Typhlocirolana* species from central and southern Morocco (LEP, ERR, ZAT, OUR, SOU, GIN, TIZ), poorly supported in Fig. 2, are split into two groups but with unsolved positions.

The position of *M. delamarei*, in the same clade as *T. haouzensis* ('central group', Fig. 2), is not clear and its generic status is not supported by the low genetic divergence from *T. haouzensis*, as also calculated by the divergence sequence data of the COXI gene. The molecular divergence investigated by COXI reveals a genetic distance of 14% between the two taxa, more similar to the distance between close species of *Sphaeromides* (*S. s. virei* and *S. v. mediodalmatina* = 12%) than to the divergence value of 29% recorded among different genera, such as *T. haouzensis* or *M. delamarei* and *S. virei*.

Application of a molecular clock provides a time framework for the evolutionary events that characterized the stygobitic cirolanids analysed here over a time scale of 180 myr up to present. The separation of the different species of Cirolanidae from a common ancestor appears to date back to the late Jurassic/lower

Table 2. Cirolaninae analysed in this study are marked with an asterisk (modified from Iliffe and Botosaneanu, 2006). The groups are sensu Botosaneanu (1986).

group	mediterranean taxa	non-mediterranean taxa
Sphaeromides	*Sphaeromides**	*Speocirolana**
	*Typhlocirolana**	*Antrolana**
	*Marocolana**	*Bahalana*
	*Turcolana**	*Mexilana*
	Cirolanides	
	Oncilorpheus	
Faucheria	*Faucheria**	*Skotobaena*
	Sphaerolana	
	Kensleylana	
Cirolana	*Saharolana**	*Anopsilana*
	Creaseriella	
	Haptolana	

Cretaceous 180-165 mya (Fig. 4). In particular, cirolanid species belonging to the different genera (*S. v. virei* vs *T. haouzensis*/*M. delamarei*) could have separated from each other 180 mya (350-150 my 95% HPD). The time of divergence for *S. seurati*/*F. faucheri* vs the *Sphaeromides* group should date back to 165 mya (350-240 95% HPD) (Fig. 4). In the Mediterranean basin, the *Typhlocirolana* taxa belonging to the M group (Fig. 2) probably separated from the species of central and northern Morocco 90 mya (140-45 my 95% HPD).

Discussion

Combination of the 16S and 12S sequence data allowed to clarify the phylogenetic issues associated with most of the stygobitic Cirolanidae, although the taxon samplings regarding stygobitic cirolanids are not still exhaustive. However, we are confident that this study has enlightened the phylogenetic relationships among the Mediterranean taxa and it can provide interesting suggestions on how to address the morphological revisions of the family. Moreover, the introduction of new sequences in the data set provided some new information and arrangements in the topologies with respect to the data obtained in previous analyses (Baratti *et al*., 2004).

Mediterranean stygobitic cirolanids are supposed to have originated directly from marine ancestors through littoral freshwater habitats, although there is no consensus on their most direct marine ancestors, *i.e*. from the deep-sea or from shallow waters (Iliffe and Botosaneanu, 2006). The thalassoid lymnostygobitic fauna representatives, as cirolanid isopods are considered 'tethyan relicts', *i.e*. species without congeners in the open sea, appearing in areas covered by the Tethys Sea (Por, 1986; Iliffe, 2000; Boutin and Coineau, 2001). In this period (late Jurassic/early Cretaceous) the major part of the actual Mediterranean lands was still submerged. Our results suggest that cirolanid taxa descend from ancestors that already occupied the shores of the western part of the Tethys Sea before the opening of the central North Atlantic (Fig. 4, about 180-165 mya) and subsequently separated following plate tectonic movements. The North Atlantic distribution of some taxa (ANTRO, SPEOC, SPH) belonging to the same 'molecular' clade (Figs 2-3) and 'morphological group' (Botosaneanu, 1986) induces us to sustain this hypothesis. In fact, in the Middle Cretaceous (115 mya) these three genera were

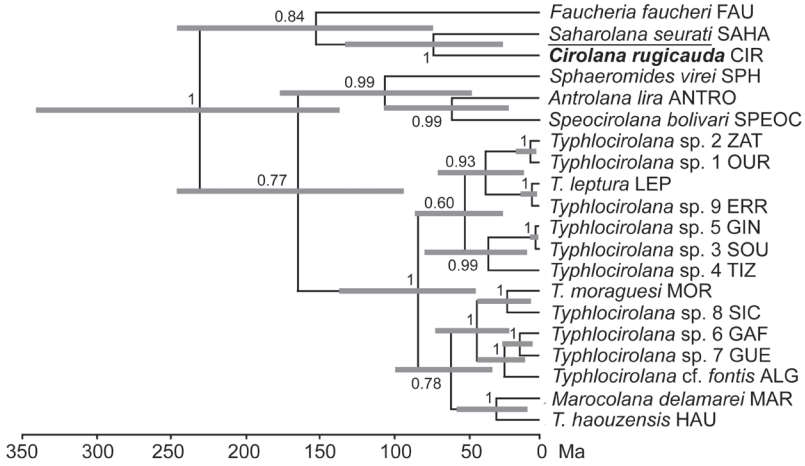

Fig. 4. Chronogram of stygobitic cirolanid isopods with divergence time estimates based on 16S sequences. Gray bars at each node show the 95% HPD interval for the date of each node. Posterior probability of node presence is indicated at branches.

separated with the opening of North Atlantic ocean. The microevolutionary history of the *Typhlocirolana* group of species appears therefore as the result of multiple vicariance events, which happened in the Mediterranean basin in the last 90-15 mya. The divergence time estimate suggests that the lineages diverged in relation to the marine transgression-regression phases and tectonic movements occurred in the Moroccan area during the Cretaceous and after the Atlas orogenesis (Eocene, 50-35 mya, Alvinerie *et al*., 1992). In the Cretaceous 90-65 mya in Morocco a deep Senonian gulf was present (Aït Boughrous *et al*., 2007) and it was generated by an atlantic big transgression which covered the Moroccan regions inhabited at present by the central-southern *Typhlocirolana* species (TIZ, GIN, SOU, ERR, LEP, ZAT, OUR). The molecular affinities among some *Typhlocirolana* taxa (M group, Figs 2-3) are probably the result of another marine regression event occured during the Tertiary period (Stoch, 2004).

The species belonging to the seven genera cluster in two groups in the phylogenetic tree, corresponding to those identified by previous morphological studies (the Faucheria and *Sphaeromides* groups). *F. faucheri* is the sister taxon of the *Sphaeromides* group. The monospecific genus *Faucheria* has morphological characteristics positioning it close to Kensleylana (Bruce and Herrando-Perèz, 2005), for which no specimen was available to us. The position of *S. seurati* outside the other groups suggests an independent origin in the subterranean waters of the Mediterranean area, as supported by some characters of incomplete adaptation to the hypogean environment (*e.g*. microphthalmy) and its crenobious biology.

In this study, some results do not correspond to previous observations based on morphological data. The species inhabiting Algeria (*T*. cf. *fontis*) and Morocco (*T. leptura* and *Typhlocirolana* from Errachidia) are split into two different clades and the last two species show a close phylogenetic relationship with species inhabiting the central Atlas, even though previous morphological investigations (Botosaneanu *et al*., 1985) suggested a close affinity between *T. leptura* and the group of taxa including *T. moraguesi*, *Typhlocirolana* from Sicily and the taxa from northern Morocco (GAF, GUE) and Algeria (ALG). The morphological affinity used in the past to sustain the close relationship of *T. leptura* with *T. moraguesi* or *T. gurney* is probably due to homoplasies among taxa inhabiting similar environments. However, uncertainties about morphological characters which could reliably distinguish different species in the genus have been expressed by some authors in relation to the high intraspecific phenotypic variability of the most important diagnostic characters (Margalef, 1958; Monod, 1930; Nourisson, 1956). The species from Algeria (*T.* cf. *fontis*, ALG) presents a clear relationship with the M group (Figs 2-3), in particular with the species inhabiting subterranean waters of northern Morocco (GAF and GUE), even though they appear quite different in important morphological characters (the shape and the length of uropods). The molecular results presented in this study suggest some discrepancies between the genetic and morphological data, which probably reflect numerous convergences and differences in the rates of morphological evolution among lineages.

Other evident contrasts between the morphological and molecular data concern the relationships between *M. delamarei* and *T. haouzensis*. The nucleotide divergence (d =14%) between the two taxa, showing high phylogenetic affinity (Fig. 2), contrasts with the conclusions from previous morphological studies (Boulanouar *et al*., 1993). Although levels of genetic divergence do not represent an absolute evaluation of taxonomic status on account of their taxon-related nature, the genetic divergences found in these taxa can be compared with those described in the literature in order to delineate a molecular threshold to distinguish species and genera in this group of taxa. The genetic distances between cirolanid species can be interpreted by comparing them with the values for other isopod taxa obtained with the same molecular marker and with the molecular thresholds described for crustaceans (Wetzer, 2001; Rivera *et al*., 2002; Taiti *et al*., 2003; Baratti *et al*., 2005; Lefebure *et al*., 2006). For Cirolanidae, a divergence range of 16% has been indicated to differentiate two different species using Cytochrome Oxidase I (Wetzer, 2001); similar values have been reported for a wide range of Crustacea (Lefebure, 2006). Therefore, the low genetic distance between *T. haouzensis* and *M. delamarei* (14% for COXI) is at the lower limit for different cirolanid species belonging to the same genus. This divergence level and the phylogenetic position of *M. delamarei* within the genus *Typhlocirolana* suggest that *M. delamarei* could fit into *Typhlocirolana* and does not require a special genus. Past morphological analyses indicated marked differences between the genera *Marocolana* and *Typhlocirolana* (Boutin, 1993; Boutin *et al*., 2002; Boulanouar *et al*., 1993); the relevant characters appeared to be adaptive features (volvation, cephalic groove of the antero-ventral margin of the first pereionite) or extremely variable within the genus *Typhlocirolana* (pleotelson form, numbers of segments and

aesthetascs of the antennulae, propodial organ presence, *etc*.). Complete or partial volvation, indicated as a synapomorphy of *Turcolana* and *Marocolana* (Boulanouar *et al*., 1993; Boutin, 1993), is probably a homoplasious character shared by five not closely related cirolanid genera (Botosaneanu, 1986).

The monophyly of the *Sphaeromides* group (sensu Botosaneanu, 1986) is confirmed by the molecular analysis, with the exception of *Turcolana*, whose membership in the *Sphaeromides* group must be revised. Unfortunately, the diversity of *Turcolana* from the other stygobitic genera of Cirolanidae in the Mediterranean basin was sustained only by the 12S sequence. Since the 16S gene sequence was lacking and the 12S phylogeny did not solve the node related to *Turcolana* sp., we cannot provide definitive phylogenetic conclusions about this taxon, and the analysis of additional specimens of *Turcolana* sp., not available at the moment, will be necessary to approach this further issue.

This study helped us to clarify some aspects of the evolutionary history of this interesting subterranean group. Current morphological keys for species identification will require a thorough re-examination in the light of our findings, followed by a complete and exhaustive morphological revision. Besides, the sampling of marine taxa belonging to the three genera *Eurydice*, *Cirolana*, *Natatolana*, at this moment known for the Mediterranean basin, would be desirable and could definitively clarify the phylogenetic relationships among Cirolanidae. In addition, samplings and genetic analyses of some cirolanid genera inhabiting shallow, slightly brackish or estuarine waters (as *Annina*, *Aphantolana*, some *Eurydice* or *Cirolana* species) could help to understand hypogean ecosystem colonization in this isopod group.

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References

Aït Boughrous A, Boulanouar M, Yacoubi M, Coineau N. 2007. The first Microcharon (Crustacea, Isopoda, Microparasellidae) from the Moroccan North Saharan platform. Phylogeny, origin and palaeobiogeography. *Contributions to Zoology* 76: 21-34.

Alvinerie J, Antunes MT, Cahuzac B, Lauriat-Rage A, Montenat C, Pujol C. 1992. Synthetic data on the paleogeographic history of Northeastern Atlantic and Betic-Rifian basin during the Neogene (from Brittany, France, to Morocco). *Palaeogeography*, *Palaeoclimatology*, *Palaeoecology* 95: 263-286.

Baratti M, Burchi A, Messana G, Yacoubi-Khebiza M. 1999. Inferring phylogenetic history of five taxa of the genus *Typhlocirolana* (Isopoda, Cirolanidae) by 12S sequence. Preliminary data. *Mémoires de Biospéologie* 26: 59-64.

Baratti M, Yacoubi Khebiza M, Messana G. 2004. Microevolutionary processes in the stygobitic genus *Typhlocirolana* (Isopoda Flabellifera Cirolanidae) as inferred by partial 12S and 16S rDNA sequences. *Journal of Zoological Systematics and Evolutionary Research* 42: 27-32.

Baratti M, Goti E, Messana G. 2005. Phylogeography of the marine isopod *Sphaeroma terebrans* (Crustacea Isopoda Sphaeromatidae) as inferred by mitochondrial DNA analysis. *Journal of Experimental Marine Biology and Ecology* 315: 225-234.

Botosaneanu L. 1986. *Stygofauna mundi: A faunistic, distributional, and ecological synthesis of the world fauna inhabiting subterranean waters*. Leiden: Brill.

Botosaneanu L, Boutin C, Henry JP. 1985. Deux remarquables cirolanides stygobies nouveaux du Maroc et de Rhodes. *Stygologia* 1: 186-207.

Boulanouar M, Boutin C, Coineau N. 1993. Un cirolanide remarquable du Maroc, *Marocolana delamarei* (Crustacé, Isopode): description, écologie et biogéographie. *Mémoires de Biospéologie* 20: 39-48.

Boutin C. 1993. *Biogéographie historique des Crustacés Malacostracés stygobies du Maroc*. Thèse de doctorat, Université de Lyon.

Boutin C, Coineau N. 2000. Evolutionary rates and phylogenetic age in some stygobiontic species. Pp. 433-451 in: Wilkens H, Culver DC, Humphreys WF, eds., *Subterranean Ecosystems - Ecosystems of the World 30*. Amsterdam: Elsevier.

Boutin C, Boulanouar M, Coineau N, Messouli M. 2002. Biodiversity in the stygobiontic cirolanids (Crustacea: Isopoda) from the Mediterranean Basin. I. A new species of *Typhlocirolana* in Morocco, taxonomic, ecological and biogeographic data. *Journal of Natural History* 36: 797-817.

Bruce NL. 1986. Cirolanidae (Crustacea: Isopoda) of Australia. *Record of the Australian Museum, Suppl*. 6: 1-239.

Bruce NL, Herrando-Perèz S. 2005. *Kensleylana briani*, a new genus and species of freshwater cave-dwelling cirolanid (Crustacea: Isopoda) from Spain. *Proceedings of the Biological Society of Washington* 118: 74-83.

Buhay JE. 2009. 'COI-like' sequences are becoming problematic in molecular systematic and DNA barcoding studies. *Journal of Crustacean Biology* 29: 96-110.

Buhay JE, Moni G, Mann N, Crandall KA. 2007. Molecular taxonomy in the dark: evolutionary history, phylogeography, and diversity of cave crayfish in the subgenus *Aviticambarus*, genus *Cambarus*. *Molecular Phylogenetics and Evolution* 42: 435-448.

Carlini DB, Manning J, Sullivan PG, Fong DW. 2009. Molecular genetic variation and population structure in morphologically differentiated cave and surface populations of the freshwater amphipod *Gammarus minus*. *Molecular Ecology* 18: 1932-1945.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* 7: 214.

Fakher El Abiari A, Oulbaz Z, Messouli M, Coineau N. 1999. A new species of *Pseudoniphargus* (Crustacea, Amphipoda) from subterranean water of northeastern Morocco: historical biogeography and evolutionary aspects. *Contributions to Zoology* 68: 161-171.

Felsenstein J. 2005. *PHYLIP (Phylogeny Inference Package) version 3.6*. Distributed by the author. Seattle: Department of Genome Sciences, University of Washington.

Finston TL, Johnson MS, Humphreys WF, Eberhard SM, Halse SA. 2007. Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. *Molecular Ecology* 16: 355-65.

Foulquier A, Malard F, Lefebure T, Douady CJ, Gibert J. 2008. The imprint of Quaternary glaciers on the present-day distribution of the obligate groundwater amphipod *Niphargus virei* (Niphargidae). *Journal of Biogeography* 35: 552-564.

Hansen HJ. 1905. Revision of the European marine forms of the Cirolaninae, a subfamily of Crustacea Isopoda. *Journal of the Linnean Society, Zoology* 29: 337-373.

Held C. 2001. No evidence for slow-down of molecular substitution rates at subzero temperatures in Antarctic serolid isopods Crustacea, Isopoda, Serolidae. *Polar Biology* 24: 497-501.

Herrando-Pérez S, Messana G, Sket B. 2007. Crustáceos del Ullal De Miravet (Castellón, España): viajeros en el espacio y en el tiempo. *Berig* 8: 4-10.

Holsinger JR. 2000. Ecological derivation, colonization and speciation. Pp. 399-415 in: Wilkens H, Culver DC, Humphreys WF, eds., *Subterranean Ecosystems - Ecosystems of the World 30*. Amsterdam: Elsevier.

Huelsenbeck JP, Ronquist FR. 2001 MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17: 754-755.

Humphreys WF. 2000. Relict faunas and their derivation. Pp. 417-432 in: Wilkens H, Culver DC, Humphreys WF, eds., *Subterranean Ecosystems - Ecosystems of the World 30*. Amsterdam: Elsevier.

Iliffe TM. 2000. Anchialine cave ecology. Pp. 59-76 in: Wilkens H, Culver DC, Humphreys WF, eds., *Subterranean Ecosystems - Ecosystems of the World 30*. Amsterdam: Elsevier.

Iliffe TM, Botosaneanu L. 2006. The remarkable diversity of subterranean Cirolanidae (Crustacea: Isopoda) in the peri-Caribbean and Mexican realm. *Bulletin de L'Institute Royal des Sciences Naturelles de Belgique, Biologie* 76: 5-26.

Jaume D, Christenson K. 2001. Amphi-Atlantic distribution of the subterranean amphipod family Metacrangonyctidae (Gammaridea). *Contributions to Zoology* 70: 99-125.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ. 1998. Multiple sequence alignment with Clustal X. *Trends in Biochemical Science* 23: 403-405.

Keable SJ. 2006. Taxonomic revision of *Natatolana* (Crustacea: Isopoda: Cirolanidae). *Records of the Australian Museum* 58: 133-244.

Ketmaier V, Argano R, Caccone A. 2003. Phylogeography and molecular rates of subterranean aquatic Stenasellid Isopods with a peri-Tyrrhenian distribution. *Molecular Ecology* 12: 547-555.

Kitaura J, Wada K, Nishida M. 1998. Molecular phylogeny and evolution of unique mud-using territorial behavior in ocypodid crabs (Crustacea: Brachyura: Ocypodidae). *Molecular Biology and Evolution* 15: 626-637.

Kluge AG, Farris JS. 1969. Quantitative phyletics and the evolution of anurans. *Systematics Zoology* 18: 1-32.

Kumar S, Tamura K, Jakobsen IB, Nei M. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics* 17: 1244-1245.

Lefebure T, Douady CJ, Gouy M, Gibert J. 2006. Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Molecular Phylogenetics and Evolution* 40: 435-447.

Margalef R. 1958. Algunos crustaceos de las aguas continentales de Espana y norte de Africa. *Miscellanea Zoologica* 1: 51-60.

Monod TH. 1930. Contribution à l'ètude des 'Cirolanidae'. *Annales des Sciences Naturelles et de Zoologie* 10: 129-183.

Muraji M, Nakahara S. 2001. Phylogenetic relationships among fruit flies, Bactrocera (Diptera, Tephritidae) based on the mitochondrial rDNA sequences. *Insect Molecular Biology* 10: 549-559.

Nourisson M, 1956. Etude morphologique comparative et critique des *Typhlocirolana* (Crustaces Isopodes Cirolanidae) du Maroc et d'Algerie. *Bulletin de la Societè des Sciences Naturelles et Physique du Maroc* 36: 103-124.

Page TJ, Short JW, Humphrey CL, Hillyer MJ, Hughes JM. 2008. Molecular systematics of the Kakaducarididae (Crustacea : Decapoda : Caridea). *Molecular Phylogenetics and Evolution* 46: 1003-1014.

Palumbi S, Martin A, Romano S, McMillan WO, Stice L, Grabowski G. 1991. *The simple fool's guide to PCR, ver. 2.0*. Honolulu: University of Hawaii.

Por FD. 1986. Crustacean biogeography of the late middle Miocene middle eastern landbridge. Pp. 69-84 in: Gore RH, Heck KL, eds., *Crustacean biogeography*. Rotterdam: Balkema.

Porter ML, Cronin TW, McClellan DA, Crandall KA. 2007. Molecular characterization of crustacean visual pigments and the evolution of pancrustacean opsins. *Molecular Biology and Evolution* 24: 253-268.

Posada D, Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14 : 817-818.

Rambaut A, Drummond AJ. 2003. *Tracer, version 1.2*. Available at: http://evolve.zoo.ox.ac.uk/software.html.

Rivera MAJ, Howarth FG, Taiti S, Roderick GK. 2002. Evolution in Hawaiian cave-adapted isopods (Oniscidea: Philosciidae): vicariant speciation or adaptive shifts? *Molecular Phylogenetics and Evolution* 25: 1-9.

Roehrdanz RL. 1993. An improved primer for PCR amplification of mitochondrial DNA in a variety of insect species. *Insect Molecular Biology* 2: 89-91.

Rosenbaum G, Lister G, Duboz C. 2002. Reconstruction of the tectonic evolution of the western Mediterranean since the Oligocene. *Journal of the Virtual Explorer* 8: 107-130.

Saitou N, Nei M. 1987. The Neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-425.

Salzat A, Bouchon D. 2000. Phylogenetic analysis of mitochondrial LSU rRNA in oniscids. *Comptes rendus de l'Académie des sciences - Series III - Sciences de la Vie* 323: 827-837.

Schmidt HA, Strimmer K, Vingron M, von Haeseler A. 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis

using quartets and parallel computing. *Bioinformatics* 18: 502-504.

Schubart CD, Cannicci S, Vannini M, Fratini S. 2006. Molecular phylogeny of grapsoid crabs from East Africa based on two mitochondrial genes and a proposal for refraining from current superfamily classification. *Journal of Zoological Systematics and Evolutionary Research* 44: 193-199.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87: 651-701.

Song H, Buhay JE, Whiting MF, Crandall KA. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences, USA* 105: 13486-13491.

Stoch F. 2004. Colonization. Pp. 235-238 in: Gunn, J ed., *Encyclopedia of cave and karst science*. London: Fitzroy Dearborn Publishers.

Strimmer K, von Haeseler A. 1997. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. *Proceedings of the National Academy of Sciences, USA* 94: 6815-6819.

Swofford DL. 2001. *PAUP-Phylogenetic analysis using parsimony*. Champaign (IL): Illinois Natural History Survey.

Taiti S, Arnedo MA, Lew SE, Roderick GK. 2003. Evolution of terrestriality in Hawaiian species of the genus Ligia Crustacea, Oniscidea. *Molecular Phylogenetetics and Evolution* 251: 1-9.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512-526.

Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Pp. 57-86 in: Miura RM, ed., *DNA sequence analysis*. Providence (RI): American Mathematical Society.

Taylor DJ, Hebert PD, Coulbourne JK. 1996. Phylogenetics and evolution of the Daphnia longispina group (Crustacea) based on 12S rDNA sequence and allozyme variation. *Molecular Phylogenetics and Evolution* 5: 495-510.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTALX Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 24: 4876-4882.

Trajano E. 2005. Evolution of lineages. Pp. 230-234 in: Culver DC, White B. eds., *Encyclopedia of caves*. Amsterdam: Elsevier.

Verovnik R, Sket B, Trontelj P. 2004. Phylogeography of subterranean and surface populations of water lice *Asellus aquaticus* (Crustacea: Isopoda). *Molecular Ecology* 13: 1519-1532.

Verovnik R, Sket B, Trontelj P. 2005. The colonization of Europe by the freshwater crustacean *Asellus aquaticus* (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity. *Molecular Ecology* 14: 4355-4369.

Wägele JW. 1989. Evolution und Phylogenetisches System der Isopoda. *Zoologica* 140: 1-262.

Wetzer R. 2001. Hierarchical analysis of mtDNA variation and the use of mtDNA for isopod systematics. *Contributions to Zoology* 70: 23-39.

Yang Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution* 10: 1396-1401.

Zakšek V, Sket B, Trontelj P. 2007. Phylogeny of the cave shrimp *Troglocaris*: evidence of a young connection between Balkans and Caucasus. *Molecular Phylogenetics and Evolution* 42: 223-235.

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