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

Mariella Baratti¹, Mariateresa Filippelli¹, Francesco Nardi², Giuseppe Messana¹

¹ Institute for the Study of Ecosystems, CNR, Via Madonna del Piano 10, Sesto Fiorentino (FI) 50019-1, Italy
² Department of Evolutionary Biology, University of Siena, Via A. Moro 2, Siena, 53100-1, Italy

E-mail: baratti@ise.cnr.it

Key words: 12S, 16S, Cirolanidae, cytochrome oxidase I, mitochondrial DNA, stygofauna

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 limno-stygobionts. 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, Saharolina 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.

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 limno-stygobionts 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,
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 Paulian and Delamare Deboutteville, 1956, Createrella 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 Speocirolanides 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 microphtalmic.

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; Noursinson 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 Speocirolanida bolivari from America are excluded. Abbreviations as in Table 1.](image-url)
Contributions to Zoology, 79 (2) – 2010

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).

<table>
<thead>
<tr>
<th>sample population</th>
<th>abbreviation</th>
<th>locality</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>16S gene</td>
</tr>
<tr>
<td>Antrolana lira Bowman, 1964</td>
<td>ANTRO</td>
<td>Virginia (USA)</td>
<td>EF460862</td>
</tr>
<tr>
<td>*Cirolana rugicada Heller, 1861</td>
<td>CIR</td>
<td>South of Luderitz (Namibia)</td>
<td>AF259544</td>
</tr>
<tr>
<td>Faucheria faucheri (Dollfus and Viré, 1900)</td>
<td>FAU</td>
<td>Avencas, Montpellier (France)</td>
<td>DQ373040</td>
</tr>
<tr>
<td>Marocolana delamarei Boutin, 1993</td>
<td>MAR</td>
<td>Marrakech (Morocco)</td>
<td>DQ194363</td>
</tr>
<tr>
<td>Saharaolana seuratii Monod, 1930</td>
<td>SAHA</td>
<td>Chott el Jerid (Tunisia)</td>
<td>DQ194359</td>
</tr>
<tr>
<td>Specirolana bolivari (Rioja, 1953)</td>
<td>SPEOC</td>
<td>Tamaulipas (Mexico)</td>
<td>EF460864</td>
</tr>
<tr>
<td>Sphaeromides virei virei (Brian, 1923)</td>
<td>SPH</td>
<td>Labin (Croatia)</td>
<td>DQ194360</td>
</tr>
<tr>
<td>S. virei mediotalmatina Sket, 1964</td>
<td>ZPH</td>
<td>Žegar Dalmatinski (Croatia)</td>
<td>AY998014</td>
</tr>
<tr>
<td>Typhlocirolana haouzensis Boutin, Boulanouar, Coineau and Messouli, 2002</td>
<td>HAU</td>
<td>Marrakech (Morocco)</td>
<td>AF356847</td>
</tr>
<tr>
<td>T. moraguesi Racovitza, 1905</td>
<td>MOR</td>
<td>Balearic Is. (Spain)</td>
<td>AF356849</td>
</tr>
<tr>
<td>T. leptura Botosaneanu, Boutin and Henry, 1985</td>
<td>LEP</td>
<td>Marrakech (Morocco)</td>
<td>DQ194365</td>
</tr>
<tr>
<td>T. cf. fontis</td>
<td>ALG</td>
<td>Mont Tlemcen (Algeria)</td>
<td>FJ460466</td>
</tr>
<tr>
<td>T. sp. 1</td>
<td>OUR</td>
<td>River Ourika (Morocco)</td>
<td>AF356850</td>
</tr>
<tr>
<td>T. sp. 2</td>
<td>ZAT</td>
<td>River Zat (Morocco)</td>
<td>AF356852</td>
</tr>
<tr>
<td>T. sp. 3</td>
<td>SOU</td>
<td>Souss (Morocco)</td>
<td>DQ194362</td>
</tr>
<tr>
<td>T. sp. 4</td>
<td>TIZ</td>
<td>River Tiznit (Morocco)</td>
<td>DQ194364</td>
</tr>
<tr>
<td>T. sp. 5</td>
<td>GIN</td>
<td>Agadir Izder (Morocco)</td>
<td>DQ194361</td>
</tr>
<tr>
<td>T. sp. 6</td>
<td>GAF</td>
<td>El Gafayt (Morocco)</td>
<td>AY093577</td>
</tr>
<tr>
<td>T. sp. 7</td>
<td>GUE</td>
<td>Guercif (Morocco)</td>
<td>AY093578</td>
</tr>
<tr>
<td>T. sp. 8</td>
<td>SIC</td>
<td>Sicily Island (Italy)</td>
<td>AF356851</td>
</tr>
<tr>
<td>T. sp. 9</td>
<td>ERR</td>
<td>Errachidia (Morocco)</td>
<td>DQ379984</td>
</tr>
<tr>
<td>Turcolana sp.</td>
<td>TUR</td>
<td>Eskisehir (Turkey)</td>
<td>no data</td>
</tr>
</tbody>
</table>

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’–GTGCCAGCMGCCGCGGT–3’) (Schubart et al., 2006). Primers used to amplify the 16S gene portion were 16Sar (5’–CGCCCTGTTATCAAAAAACAT–3’) and 16Sbr (5’–CCG-GTCTGAACTCAGATCACACGT–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’–TTGATTTTTTGGTCATCCAGAAAGT -3’ (Roer-danz, 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 (Phylogenetic 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, Speciocolana, 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 invariant, 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. virei vs Saharolana seurati Monod, 1930). For the 16S gene, we examined 444 aligned base pairs: 99 invariant, 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 ($\chi^2= 125.65$, p<0.0001) and 16S ($\chi^2= 125.80$, 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, Speciocolana 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;
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, Waegle, 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/
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
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 Boughrouss et al., 2007) and it was generated by an Atlantic big transgression which covered the Moroccan regions inhabited at present by the central-southern regions inhabited at present by the central-southern

The molecular affinities among some Typhlocirolana taxa (M group, Figs 2-3) are probably the result of another marine regression event occurred 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 Spheromides groups). F. faucherii is the sister taxon of the Spheromides 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. microphthalmmy) 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 (Boulouanour 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 Cirolanididae, 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; Boulouanour 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 antennae, propodial organ presence, etc.). Complete or partial volvation, indicated as a synapomorphy of *Turcolana* and *Marocolana* (Boulouanour et al., 1993; Boutin, 1993), is probably a homoplasicious character shared by five not closely related cirolanid genera (Botsosaneau, 1986).

The monophyly of the *Sphaeromides* group (sensu Botsosaneanu, 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 *Aminia*, *Aphantolana*, some *Eurydice* or *Cirolana* species) could help to understand hypogean ecosystem colonization in this isopod group.

**Acknowledgements**

Many thanks to B. Sket and M. Tanatmis for providing *Turcolana* and *Sphaeromides* specimens; to B. Hutchins for providing the American Cirolanidae; to F. Bréhier for providing *Faucheria* specimens; to Jos Notenboom for the critical review of the manuscript; and to M. Messouli, M. Yacoubi and M. Boulanouar for the help with field work and for interesting discussions.

**References**


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


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.


