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Microevolutionary processes in the stygobitic genus *Typhlocirolana* (Isopoda Flabellifera Cirolanidae) as inferred by partial 12S and 16S rDNA sequences

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Abstract

Morocco is one of the regions of the world where many interesting discoveries have recently been made in the field of stygobiology, particularly concerning the cirolanid isopod fauna. One of the most interesting, variable and wide spread of these taxa is the perimediterranean stygobitic genus *Typhlocirolana* Racovitza, 1905, which has colonized the continental groundwater of Israel, Sicily, Spain, the Balearic Islands, Algeria and Morocco with several species. More populations have recently been found in Morocco, in some southern regions around Agadir, in High Atlas valleys near Marrakech and in the northeastern part of the country close to Oujda. The populations of these zones are not yet described and are the subject of this molecular analysis, together with other already designated species. To investigate the phylogenetic relationships and evolutionary history of the *Typhlocirolana* populations inhabiting the western Mediterranean basin, we analysed DNA sequences from the mitochondrial 12S and 16S rDNA genes. The molecular data were also used to infer the mechanisms driving the evolution of this thalassoid limnostygobitic cirolanid taxon, considered a good paleogeographic indicator because of its poor dispersion abilities. Vicariance because of paleogeographic events in the western Mediterranean basin played a prime evolutionary role in the Cirolanidae, as already suggested by morphological and ecological studies. Application of a molecular clock provided a time framework of the microevolutionary events occurring in *Typhlocirolana* populations over the last 40 myr.

Key words: Subterranean aquatic fauna – mitochondrial genes (12S and 16S) – western Mediterranean basin – cirolanid isopods

Introduction

Subterranean ecosystems are simplified, but not simple, systems considered as optimal natural laboratories to study the evolution of subterranean taxa and to investigate the effects of spatial and temporal isolation on genetic divergence (Culver et al. 1995).

Morocco is one of the regions of the world where many interesting discoveries have recently been made in the field of stygobiology. Among the many groups of Crustacea discovered in the area, there are several new amphipod and isopod taxa whose presence, origin and distribution have been described (Coineau 1994; Fakher El Abiari et al. 1999; Coineau et al. 2001).

Morocco is revealing an increasing richness in cirolanid isopods. One of the most interesting, variable and wide spread of these taxa is the perimediterranean stygobitic genus *Typhlocirolana* Racovitza, 1905, which has colonized the continental groundwater of Israel, Sicily, Spain, the Balearic Islands, Algeria and Morocco with several species.

The subterranean species of this genus have been divided into three major groups on the basis of morphological analyses: the *leptura* group, type species *T. leptura* Botosaneanu, Boutin & Henry, 1985, from the surroundings of Marrakech, thin and long, with a still primitive chaetotaxy; the *fontis* group, type species *T. fontis* (Gurney 1908) from Algeria, to which belongs *T. haouzensis* Boutin, Boulanouar, Coineau & Messouli, 2002, from the Marrakech phreatic layer, showing several plesiomorphies; the *moraguesi* group, type species *T. moraguesi* Racovitza, 1905, from the Balearic Islands, to which belong *T. rifana* Margalef, 1958, from northern Morocco, characterized by more apomorphies than *T. haouzensis* (Boutin, in litteris).

More populations have recently been found in Morocco, in some southern regions around Agadir, in High Atlas valleys near Marrakech and in the north-eastern part of Morocco near Oujda.

The populations from these zones have not yet been attributed to any of the previous groups. They are the subject of the present molecular analysis, together with the already described species.

Molecular techniques appear to be particularly useful since sometimes the stygobitic taxa show a low morphological differentiation that can be explained either by a poor diagnostic power of the phenotypic characters used, or by convergent evolution in similar environments, a common phenomenon in troglobitic species. The resulting taxa may continue to diverge genetically in the absence of morphological differentiation, producing sibling species: this seems to be the case of *T. moraguesi* and *Typhlocirolana* sp. from Sicily (Caccone et al. 1986) and similar situations have been reported for other hypogean isopod genera such as *Oritoniscus*, *Stenasellus* and *Trichoniscus* (Sbordoni et al. 2000).

Following a preliminary investigation of the phylogenetic relationships among perimediterranean taxa of cirolanid isopods (Baratti et al. 1999), we analysed DNA sequences from the mitochondrial 12S and 16S rDNA genes in several *Typhlocirolana* populations. The usefulness of these molecular markers to infer phylogeny in Crustacea at various taxonomic levels has been well demonstrated (Taylor et al. 1996; Kitaura et al. 1998; Schubart et al. 1998; Salzat and Bouchon 2000), although few data are available for subterranean species (Englisch and Koenemann 2001; Stepien et al. 2001; Wetzer 2001; Rivera et al. 2002).

The aim of this research was to investigate the phylogenetic relationships and evolutionary history of the *Typhlocirolana* populations inhabiting the western Mediterranean basin and to identify the mechanisms driving the evolution of this thalassoid limnostygobitic cirolanid taxon. Subterranean Cirolanidae are good paleogeographic indicators because of their poor dispersal abilities (Boutin and Coineau 2000). The characteristics of subterranean fauna and the current information about

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stygobitic cirolanids suggest that allopatric speciation is the common means of population differentiation. This hypothesis can be confirmed as the divergence and phylogeography of sister taxa are concordant with vicariance events.

Materials and Methods

Materials examined

Genomic DNA was extracted from specimens preserved in absolute ethanol, collected from *Typhlocirolana* populations (Fig. 1). From Morocco: Marrakech, Ecole des Mines, *T. haouzensis* (HAU); from Spain: Balearic Islands, *T. moraguesi* (MOR). Non-described taxa: from Morocco: River Zat (ZAT); River Ourika (OUR); River Souss (SOU), Tiznit (TIZ), Agadir Izder (GIN), Guefa (GAF), Guercif (GUE), Outat El Haj (HAY); from Italy: Sicily (SIC). *Euridyce affinis* (EUR) was used as outgroup (GenBank no. AJ388073) for 16S alignment, while *Marocolana* sp. (MAR) (AF356858) was used as outgroup for the 12S gene portion. Four specimens were examined for each population (Table 1).

MtDNA sequencing

PCR amplification products were obtained from the 12S and 16S mitochondrial gene portions. The protocols for DNA extraction and 12S rRNA amplification were described in Baratti et al. (1999). The protocol for 16S rRNA amplification followed Salzat and Bouchon (2000), with minor changes. PCR products were run on a 1.5% agarose gel, containing 0.5 mg/ml ethidium bromide. The amplification patterns were analysed with the Gel Analysis Program v. 3.6. (Kodak Company, Rochester, NY) PCR products were purified (ExoSAP-IT, Amersham Biosciences, Uppsala, Sweden) sequenced with a Perkin–Elmer sequencing kit and analysed with an ABI 310 automated sequencer (Applied Biosystems, Foster City, USA). It was not possible to obtain good sequences for the *Marocolana,* SOU, GIN and TIZ populations for 16S rRNA, despite repeated attempts with various reaction conditions. All the sequences are deposited in GenBank with the accession numbers reported in Table 1.

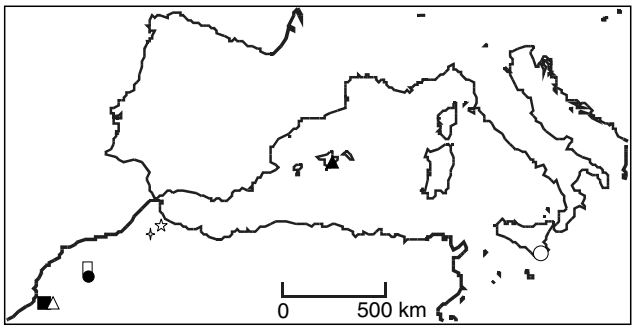

Fig. 1. Sample localities for all the populations studied. Abbreviations as in Table 1: MOR, ▲; SIC, ○; GAF and HAY, ☆; GUE, ✧; HAU, □; ZAT and OUR, ●; SOU TIZ, ■ and GIN, △

Sequence analysis and nucleotide diversity

Electroferograms were manually aligned using ESEE software v. 3.2 (Eric Cabot, 1998, Eyeball Sequence Editor) to correct apparent anomalies. To search for available matches with published sequences of other invertebrates, we also aligned the sequences on BLAST at the web site of NcbiI.

Multiple alignments were obtained by CLUSTALX (CLUSTAL X Multiple Sequence Alignment Program v. 1.81; 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 evolutionary distances were analysed with MEGA v. 2.1 (Kumar et al. 2001). The Kimura two-parameter distance was calculated, considering both Ts–Tv and Tv only and taking into account that the Ts/Tv ratio decreases as pairwise sequence divergence increases (Kimura 1980).

Phylogenetic analysis and evolutionary rates

A χ^2 test of homogeneity of base frequencies across taxa was carried out using PAUP 4.0, b. 10 (Swofford 2001). We performed the likelihood mapping method (Strimmer and von Haeseler 1997) using TREE-PUZZLE (Schmidt et al. 2002) in order to test the *a priori* phylogenetic signal in the two portions of the mtDNA studied. Testing for the evolutionary model that best fit our data was conducted with MODELTEST v. 3.04 (Posada and Crandall 1998), based on a likelihood ratio test. Different models of nucleotide substitutions were fitted to each data set. For 12S, the TRN model was the best one selected (Tamura and Nei 1993), while for 16S the TIM model was selected (Rodriguez et al. 1990). Both models were corrected for rate heterogeneity among sites with a Gamma distribution (*G*, Yang 1993). The phylogenetic reconstructions have been obtained using PAUP. Once the appropriate model was selected, maximum likelihood (ML) (Felsenstein 1981) and neighbour-joining (NJ) (Saitou and Nei, 1987) analyses were performed with all the parameter values estimated by MODELTEST. NJ trees were constructed with distances computed using the best-fit model obtained

Table 1. Populations studied and relative abbreviations with base composition and Genbank accession number (no data when sequences for a gene are lacking)

Sample population	Abbreviation	Base composition, 12S/16S				GenBank accession number	
		T	C	A	G	12S gene	16S gene
T. haouzensis	HAU	37.9/34.7	15.9/10.8	26.6/31.2	19.6/23.3	AF356855	AF356847
T. moraguesi	MOR	39.9/36.4	14.7/9.7	29.7/31.9	15.6/22.0	AF356857	AF356849
Typhlocirolana sp.							
River Ourika	OUR	37.7/35.0	15.2/11.0	26.7/28.9	20.4/25.3	AF356854	AF356850
River Souss	SOU	39.4/–	14.8/–	28.5/–	17.3/–	AF356868	No data
River Tiznit	TIZ	39.6/–	13.6/–	28.7/–	18.1/–	AF356864	No data
Agadir Izder	GIN	40.8/–	14.4/–	28.2/–	16.5/–	AF356866	No data
El Gafayt	GAF	40.2/35.5	15.4/10.1	24.8/29.7	19.6/24.7	AY093574	AY093577
Guercif	GUE	39.3/34.9	15.4/10.1	25.6/30.8	19.7/24.2	AY093575	AY093578
El Hay	HAY	39.0/35.2	15.4/10.5	25.6/29.2	20.0/25.1	AY093576	AY093579
Sicily Island	SIC	39.9/36.2	15.6/9.9	26.7/31.9	17.7/22.0	AF356856	AF356851
River Zat	ZAT	36.1/34.6	17.3/11.0	28.2/28.9	18.5/25.6	AF356853	AF356852
Marocolana delamarei	MAR	36.0/–	17.2/–	27.8/–	19.0/–	AF356858	No data
Eurydice affinis	EUR	–/28.7	–/19.6	–/32.4	–/19.3	No data	AJ388073
Average		38.8/34.7	15.4/11.3	27.3/30.5	18.5/23.6		

with MODELTEST. Parsimony analysis (MP, Kluge and Farris 1969) was carried out using the heuristic search algorithm and tree bisection TBR branch swapping. A strict consensus tree was calculated when more than one tree resulted. Branch supports were assessed by 1000 non-parametric bootstrap replicates. MP, ML and NJ analyses were performed for each set of DNA sequences separately and in a combined analysis (total evidence approach) for the taxa sequenced for both the genes.

Divergence time estimates

A relative rate test (Tajima 1993) was conducted with the MEGA software to test the molecular clock hypothesis. The zero hypothesis of this test is that the evolutionary rate is homogeneous in sister groups. When no significant differences were observed between lineages, we applied a molecular clock to estimate the time of divergence from a common ancestor. The Tajima test, as other relative χ^2 tests, for its conservative nature, is unlikely to determine low levels of rate variation with low percentage of rejection when sequence lengths lower then 1000 bp are used (Bromham et al. 2000; Kumar and Gadakar 2001). Therefore, a Pattern Homogeneity Disparity Index (I_D) test was also performed (Kumar and Gadakar 2001) with 2000 Monte-Carlo replicates in MEGA and a branch length test was carried out by LINTRE (Takezaki et al. 1995).

The clock for the 16SrRNA was calibrated using data obtained for this gene in other decapods (Cunningham et al. 1992; Schubart et al. 1998, Steelmann and Reeb 2001). Among the rates found for Decapoda, we used the intermediate value of Schubart et al. (1998) (0.65% per myr), based on Kimura two-parameter distances.

Results

After alignment, 349 base pairs were obtained for the 12S gene. Of these base pairs, 229 sites were not variable, 55 were variable uninformative, whereas 65 were parsimony informative. The percentage of divergence between populations was 2–20%, while at the genus level the average genetic distance was 23% (Table 2).

For the 16S gene, we examined 467 base pairs, among which 146 were variable sites with 115 parsimony informative sites. For this gene, the range of divergence between populations was 2–25%, whereas at the genus level the average distance was 38%. No or very low genetic differentiation within each population was detected for either rRNA gene (Table 3).

All the sequences are A–T rich (mean A−T content = 66.1% for 12S, 65.2% for 16S; Table 1). This is in agreement with the observation that arthropod mitochondrial genomes generally tend to be highly A + T biased, although the A–T content is lower than in other arthropods such as insects (Simon et al. 1994; Muraji and Nakahara 2001).

Results obtained by the chi-square homogeneity test showed homogeneous base composition within the ingroup taxa for the 12S ($\chi^2 = 12.404153$, p=0.99956) and 16S ($\chi^2 = 5.380$, $p = 0.99975$) rRNAs.

The results of the likelihood mapping method carried out by TREE-PUZZLE suggested the presence of a stronger phylogenetic signal in 16S as compared to 12S.

The NJ analysis of the 12S gene revealed two sister clades, a 'Mediterranean' and an 'Atlantic' one (Fig. 2), as confirmed by the ML analysis (data not given).

The first clade includes the northern Moroccan populations (GUE–HAY–GAF), the Mediterranean *T. moraguesi* and the undetermined Sicilian species. This group is well separated from the others in all phylogenetic analyses and for both genes (Figs 2 and 3). In the tree topology, the northern Moroccan populations are grouped together whereas the MOR–SIC populations are clustered as their sister taxa. The second group, including the populations from the western side of the Atlas (TIZ–SOU–GIN and ZAT–OUR), is not maintained in the parsimony analysis of 12S. The taxon *T. haouzensis* always appears as a sister group of all the populations examined here. Two

Table 2. Kimura two-parameter distances in percentage based on 12S sequences: above the diagonal K2P TV only, below TS + TV

	HAY	GUE	GAF	MOR	SIC	SOU	TIZ	GIN	ZAT	OUR	HAU
HAY	**	0.02	0.03	0.05	0.05	0.07	0.07	0.06	0.09	0.07	0.05
GUE	0.02	**	0.01	0.04	0.04	0.06	0.06	0.05	0.08	0.07	0.04
GAF	0.08	0.06	**	0.04	0.05	0.06	0.07	0.05	0.08	0.07	0.04
MOR	0.16	0.15	0.14	**	0.04	0.08	0.09	0.08	0.10	0.09	0.07
SIC	0.16	0.14	0.14	0.13	**	0.08	0.08	0.08	0.10	0.10	0.07
SOU	0.15	0.13	0.13	0.16	0.14	**	0.02	0.05	0.07	0.06	0.06
TIZ	0.18	0.16	0.16	0.17	0.18	0.09	**	0.08	0.07	0.05	0.09
GIN	0.15	0.12	0.12	0.17	0.14	0.09	0.11	**	0.04	0.08	0.10
ZAT	0.19	0.17	0.18	0.21	0.20	0.10	0.15	0.13	**	0.07	0.09
OUR	0.17	0.15	0.15	0.19	0.18	0.13	0.13	0.10	0.05	**	0.06
HAU	0.16	0.14	0.13	0.17	0.18	0.15	0.16	0.13	0.17	0.14	**

Abbreviations as in Table 1.

Table 3. Kimura two-parameter distances based on 16S sequences: above the diagonal K2P TV only, below TS + TV

	HAY	GUE	GAF	MOR	SIC	ZAT	OUR	HAU
HAY	**	0.01	0.02	0.04	0.05	0.06	0.06	0.07
GUE	0.05	**	0.02	0.04	0.04	0.06	0.05	0.06
GAF	0.07	0.08	**	0.06	0.06	0.07	0.08	0.09
MOR	0.18	0.16	0.22	**	0.02	0.06	0.06	0.07
SIC	0.21	0.17	0.24	0.10	**	0.07	0.06	0.07
ZAT	0.24	0.20	0.26	0.18	0.19	**	0.00	0.06
OUR	0.23	0.19	0.25	0.17	0.19	0.02	**	0.06
HAU	0.25	0.19	0.28	0.21	0.18	0.19	0.18	**

Abbreviations as in Table 1.

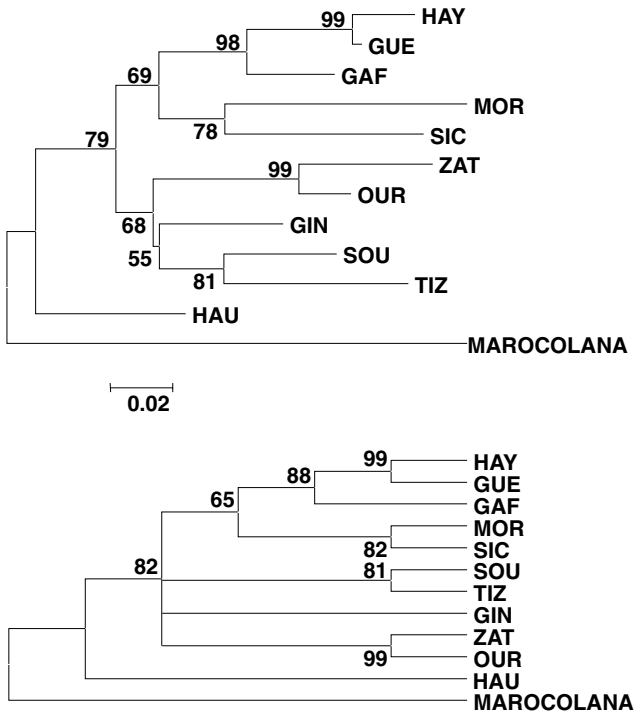

Fig. 2. Phylogenetic trees obtained by 12S rRNA sequences. Abbreviations as cited in Table 1: (a) neighbour-joining tree; (b) Maximum parsimony strict consensus tree obtained by two trees. CI = 0.72; RI = 0.65; tree length = 252. Bootstrap values are reported at each branch node obtained after 1000 replicates

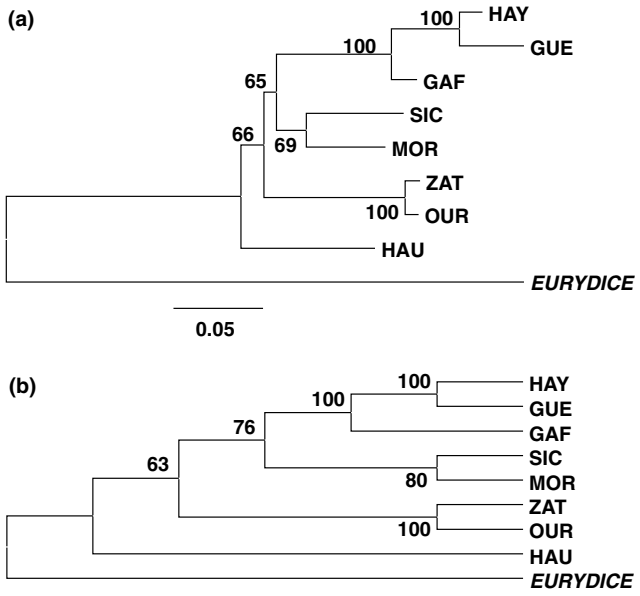

Fig. 3. Phylogenetic trees obtained by 16S rRNA sequences. Abbreviations as cited in Table 1: (a) neighbour-joining tree; (b) maximum parsimony strict consensus tree obtained by two trees. CI = 0.82; RI = 0.69; tree length = 326. Bootstrap values are reported at each branch node obtained after 1000 replicates

parsimonious trees were obtained for each gene and the two strict consensus trees were calculated (for 12S, CI = 0.72, RI = 0.65, tree length, 252; for 16S, CI = 0.82, RI = 0.69, tree length, 326). The Mediterranean group is consistently maintained in the parsimony consensus tree of 12S (Fig. 2). The lack of sequences for some taxa did not allow us to confirm this clustering for the 16S rRNA. Moreover, the ML analyses performed on the 16S data set (omitted), produced an unresolved topology.

The total approach did show results perfectly matching those obtained with the two separated data sets of the common sequences.

The Tajima test showed rate homogeneity between taxa and the molecular clock hypothesis was never rejected ($P > 0.05$). The disparity index test revealed a moderate heterogeneity scenario, with $I_D > 0$ at the significant limit of 0.05 between the couples SIC–MOR and ZAT–OUR, besides all the other values were not significant. This situation was not confirmed by branch length test. On the other part, low branch variation is often observed in order to the stochastic nature of sequence change at the nucleotide level, also in closely related taxa (Hedges and Kumar 2003). These considerations allowed us to calculate the times of divergence from the common ancestor of taxa in the clades (Table 4). With a rate of divergence of 0.65% per myr, the largest separation is between ZAT–OUR and the 'Mediterranean' group (34 myr).

Table 4. Ranges of divergence since common ancestor based on Kimura two-parameter (K2P) distance and crustacean molecular-clock calibrations (intermediate value for Crustacea from Schubart et al. 1998, for grapsid crabs: 0.65% K2P/myr)

Common ancestor of	Distance	Myr
1. (HAY–GUE)–GAF	7	11
2. MOR–SIC	10	15
3. HAU–(ZAT–OUR)	16	25
4. (HAY–GAF–GUE)–(MOR–SIC)	20	30
5. (ZAT–OUR)–'Mediterranean group'	22	34
6. HAU–'Mediterranean group'	24	37

Discussion

The identification of species boundaries is often difficult and requires a multidisciplinary study when the populations live in allopatry. The first question concerning the taxonomic status of the new populations from Morocco is: can we consider some of them as new species? The samples of *Typhlocirolana* described as good species (*T. moraguesi* and *T. haouzensis*) show an average divergence from the other taxa of 13–18% for 12S and 18–25% for 16S (Tables 2 and 3). The divergence values among the *Typhlocirolana* populations range from 2 to 25% for the 16S gene and from 2 to 20% for the 12S rDNA portion. We can also compare the levels of molecular sequence divergence detected in our study with patterns of genetic differentiation found in many other crustacean taxa. The divergence values cited in the literature for well differentiated, undisputed species range from 4 to 5% in Crustacea (France and Kocher 1996). Similar values were found for all the populations in our study, apart from the pair ZAT–OUR (2–5%) and the populations from the northern Atlas HAY–GUE (2–5%). The comparison SOU–TIZ also shows a low value if we consider TV only.

The low level of genetic divergence between the populations ZAT–OUR or GUE–HAY can be explained by the hydrologic basin in which they live. In aquatic fauna, population differentiation is related more to 'hydrologic relationships',

a measure of subterranean connectivity, than to geographic distance (Sbordoni et al. 2000). In fact, populations from different caves in the same hydrologic system often represent a single panmictic gene pool, showing low or no genetic differentiation. Moreover, many hypotheses maintain that low levels of divergence can be correlated with single, recent invasions of inland waters, while highly differentiated taxa indicate multiple cladogenetic events (Caccone et al. 1986; Holsinger 2000).

A central aim of this paper was to clarify the phylogenetic relationships and reconstruct the cladogenetic events of Mediterranean taxa belonging to the genus *Typhlocirolana*. It was interesting to test whether vicariance because of paleogeographic events in the western Mediterranean basin played a prime evolutionary role in the Cirolanidae, as suggested on the basis of their morphology and ecology (Boutin and Coineau 2000).

The interruption of gene flow between the inland water colonizers and the interstitial populations inhabiting the marine coastal sands was probably caused by vicariance during the Tethys regression (Turonian, 90 myr; Boutin et al. 2002).

Application of a molecular clock (Schubart et al. 1998) provides a time framework of the microevolutionary events occurring in *Typhlocirolana* populations over the last 40 myr (Table 4). The northern part of Morocco was submerged under the Tethys Sea till the upper Miocene (Tortonian-Messinian 10–6 myr) and all the known stygobitic taxa (Isopoda Microparasellidae and Microcerberidae; Amphipoda Melitidae) are distributed in formerly submerged areas (Coineau and Boutin 1996). At the end of the Eocene (40–30 myr) the phases of Atlas orogenesis began (Alvinerie et al. 1992) and probably led to separation between the northern group and the other populations (37–34 myr; Table 4, rows 5 and 6). The populations constituting the Mediterranean group, that is, the northern Morocco populations of *Typhlocirolana*, *T. moraguesi* and *Typhlocirolana* sp. from Sicily could have separated when the hydrological continuity was interrupted in the Aquitanian (30–20 myr), with fragmentation of the Alboran–Kabylian–Calabrian Plate (30 myr; Table 4, row 4) (Biju-duval et al. 1976; Rögl and Steininger 1983; Rögl 1998).

Caccone et al. (1986) suggested that the genetic variability in *T. moraguesi* and *Typhlocirolana* sp. from Sicily was because of a 'recent' colonization (4 myr) of inland waters in relation to the sedimentary cycles of the upper Miocene–middle Pliocene. However, our genetic data suggest that the cladogenetic events occurring in the two taxa MOR and SIC should be pushed back in time (15 myr; Table 4, row 2).

The present distribution of *Typhlocirolana* (according to the new data presented here) has common characteristics with that of other crustaceans present in subterranean waters of Morocco and the Mediterranean basin, for example, the isopod genus *Microcharon* with its species *M. alamiae* Boulanouar et al., 1997, belonging to the eastern Mediterranean '*motasi*' group, the species of the '*messouli*' group and the situation of the isopod genus *Microcerberus* (Coineau 1994; Coineau et al. 2001).

Our understanding of the evolutionary events in the *Typhlocirolana* group could be increased by extending the molecular studies to other populations inhabiting Moroccan subterranean waters (possibly including populations of the *leptura* group) and the eastern Mediterranean basin, and by comparing the data with the results of an exhaustive morphological study and re-evaluation of the diagnostic characters.

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Zusammenfassung

Mikroevolutionäre Prozesse in der stygobiontischen Gattung Typhlocirolana (Isopoda, Flabellifera, Cirolanidae) abgeleitet aus partiellen 12S rDNA- und 16S rDNA-Sequenzen

Marokko ist eine Region der Welt, in der auch gerade jetzt viele interessante Entdeckungen auf dem Gebiet der Stygobiologie, besonders in Bezug auf die cirolanide Isopodenfauna, gemacht werden konnten. Eines der interessantesten und weit verbreitetsten Taxons der cirolaniden Isopoden ist die perimediterrane, stygobiontische Gattung Typhlocirolana Racovitza, 1905, die das kontinentale Grundwasser von Israel, Sizilien, Spanien, den Balearen, sowie von Algerien und Marokko mit mehreren Arten bewohnt. Weitere Populationen wurden vor kurzem in Marokko, südlich von Agadir, in den Tälern des Hohen Atlas bei Marrakesch und in nordöstlichen Teilen des Mittleren Atlas nahe bei Oujada gefunden. Die Populationen dieser Regionen wurden bisher noch nicht beschrieben und sind, zusammen mit bereits bestimmten Arten, Gegenstand der vorliegenden molekularen Analyse. Um die phylogenetischen Beziehungen und den Verlauf der Evolution der Typhlocirolana-Populationen zu untersuchen, haben wir DNA-Sequenzen der mitochondrialen 12S- und 16-S-rDNA-Gene analysiert. Die molekularen Daten wurden auch verwendet, um die bestimmenden Mechanismen in der Evolution dieser meerwasser-süßwasser-circolaniden Stygobionten-Taxa zu erfassen, die, wegen ihrer geringen Dispersionsfhigkeit, als gute paläogeographische Indikatoren angesehen werden. Vikarianz, bedingt durch die paleogeographischen Ereignisse im Mittelmeerbecken, spielte in der Evolution der Cirolanidae die primre Rolle, was bereits auf Grund morphologischer und ökologischer Untersuchungen angenommen worden war. Durch den Einsatz der molekularen Uhr-Hypothese konnte ein Gerüst der mikroevolutiven Ereignisse, die sich bei den Typhlocirolana-Populationen in den letzten 40 Millionen Jahren ereignete haben, rekonstruiert werden.

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