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DNA barcodes, cryptic diversity and phylogeography of a W Mediterranean assemblage of thermosbaenacean crustaceans

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We assess the occurrence of crypticism and analyse the phylogeography of a thermosbaenacean crustacean, the monodellid *Tethysbaena scabra*, endemic to the Balearic Islands (W Mediterranean). This species occurs only in mixohaline waters of coastal wells and caves adjacent to the seashore. We have used the mitochondrial DNA barcode region to assess its genetic population structure throughout the anchialine environment of the islands. Maximum likelihood phylogenetic analyses showed that the Balearic *Tethysbaena* and those from the NW Italian Peninsula form a monophyletic assemblage subdivided into several lineages. Cytochrome c oxidase subunit 1 (*cox1*) p-distances among the more divergent Mallorcan lineages are remarkably high and on par with those established between the formally described species *T. scabra* from Menorca and *T. argentarii* from Italy. This result and the application of the generalised mixed Yule coalescence model (GMYC) suggest that at least some of the Mallorcan lineages represent cryptic species. A clear-cut phylogeographic pattern is displayed by this anchialine assemblage: six of its seven lineages appear in allopatry, with the exception of a Mallorcan lineage limited to a single cave nested within the geographic range of another lineage. All lineages show a distribution reduced to a single cave or to short portions of coast not exceeding 60 km in length. Our coalescence estimations suggest an early Tortonian (10.7 Ma) origin for the Balearic + Italy *Tethysbaena* clade, an age that is largely prior to the onset of the eustatic oscillations associated with the Quaternary glaciations. Only the diversification that took place within some of the Mallorcan lineages could be coeval with the broad glacio-eustatic oscillations of the Quaternary.

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Introduction

The anchialine environment refers to the masses of mixohaline water of aquifers affected by seawater intrusion (Stock *et al.* 1986; Bishop *et al.* 2015). This medium conforms a narrow band in coastal areas that extends inland to the limit of seawater penetration (Stock *et al.* 1986; Bishop *et al.* 2015). Its subterranean waters harbour a disproportionate number of phylogenetic and biogeographic relicts whose distribution, often limited to a single island or narrow portion of coast, renders them extremely vulnerable to natural or anthropogenic impacts (Iliffe & Kornicker 2009).

Climatic fluctuations during the Quaternary have played a major role in determining the current distribution and genetic structure of animal populations (Hewitt 2004). In coastal areas, sea-level oscillations associated with major episodes of glaciation–deglaciation have led to the recurrent exposure and retreat of emerged land. Such cycles have largely determined the distribution and phylogeography of coastal organisms through promoting the fragmentation and ulterior secondary contact of their populations, their local extinction, or sieving their genetic diversity through the establishment of population bottlenecks (Molins *et al.* 2009).

The anchialine environment represents an ideal study system where to assess the effect of past sea-level oscillations on the phylogeographic structure of species tightly associated with coastal areas. This peculiar habitat experienced dramatic changes in its extent and connectivity in the past due to the recurrent changes in sea level associated with Quaternary glacial cycles, or even to more ancient events.

The few studies performed until now on the phylogeography of anchialine taxa have dealt with diadromous species (i.e. that pass part of their life cycle in the sea, usually as dispersive larvae) and have shown that this aspect of the natural history of the organisms largely determines the spatial genetic structure of their populations (Kano & Kase 2004; Santos 2006; Craft *et al.* 2008; Russ *et al.* 2010). Only the study by Bauzà-Ribot *et al.* (2011) has focused on an unequivocally non-diadromous anchialine taxon – the amphipod crustacean *Metacrangonyx longipes* Chevreux, 1909 – and revealed that this species shows a high level of population genetic structure along the apparently continuous anchialine habitat of a Mediterranean island.

Among the members of the anchialine fauna outstand the Thermosbaenacea, an odd order of peracaridan crustaceans characterised by the development of a dorsal brood pouch in gravid females that contrasts with the ventral 'marsupium' exhibited by females of other peracarid orders (Fig. 1A). The group is strictly aquatic subterranean (stygobiont) and appears rarely in samples, with the few (35) species known thus far showing a very localised distribution in tropical to warm-temperate latitudes, mostly in anchialine habitats (Wagner 1994, 2012; Jaume 2008; Shimomura & Fujita 2009). As the rest of peracaridans, they have no free-swimming larval stages, brooding females releasing miniaturised adults instead. In addition, none of the species known has been reported to be diadromous. The dispersal abilities of thermosbaenaceans seem consequently to be extremely limited.

Here, we use DNA barcodes [667 bp of the Cytochrome oxidase I (*cox*1) mitochondrial DNA gene; Hebert *et al.* 2003a,b] to assess for the first time the phylogeography of a thermosbaenacean crustacean, the monodellid *Tethysbaena scabra* (Pretus, 1991), endemic to the Balearic Islands of Mallorca, Menorca and their peripheral islets Cabrera and Dragonera (W Mediterranean; Fig. 1B; Pretus 1991; Jaume 1993; Wagner 1994). This species has a broad distribution throughout the anchialine medium of the islands (Wagner 1994), appearing only in mixohaline waters of coastal wells and caves adjacent to the seashore and never in fresh inland groundwaters. These islands and islets have remained isolated from the continent and from the rest of the Balearic Archipelago since the beginning of the Pliocene, but with the advent of the Pleistocene, glacio-eustatic oscillations have impelled their recurrent connection and separation, modifying the size and shape of emerged land (Cuerda 1975; Pomar 1979). We use the mitochondrial DNA barcode region of this species as a population genetic marker to assess its levels of variation throughout the anchialine environment of the islands, in search of past sea-level signals that could have determined its genetic population structure.

Materials and methods

Sampling

Specimens of *Tethysbaena scabra* were collected from 12 different localities scattered over the coasts of Mallorca, Menorca and adjacent islets. In addition, specimens from eight populations covering the entire geographic range of the genus were used as outgroups. The genus currently includes 25 formally described species (Wagner 1994, 2012), and its distribution covers from the coasts of the Indian Ocean (Arabian Peninsula; own obs.) to the Edwards Aquifer in Texas (USA). We sampled the following taxa: *T. argentarii* (Stella, 1951) from its single known locality at Monte Argentario (Tuscany, Italy), *T. atlantomaroccana* (Boutin & Cals, 1985) from its type locality at the flood plain of river Tensift in Marrakech (Morocco), and six not yet formally described species from, respectively, a well placed near Tasla (Morocco; two

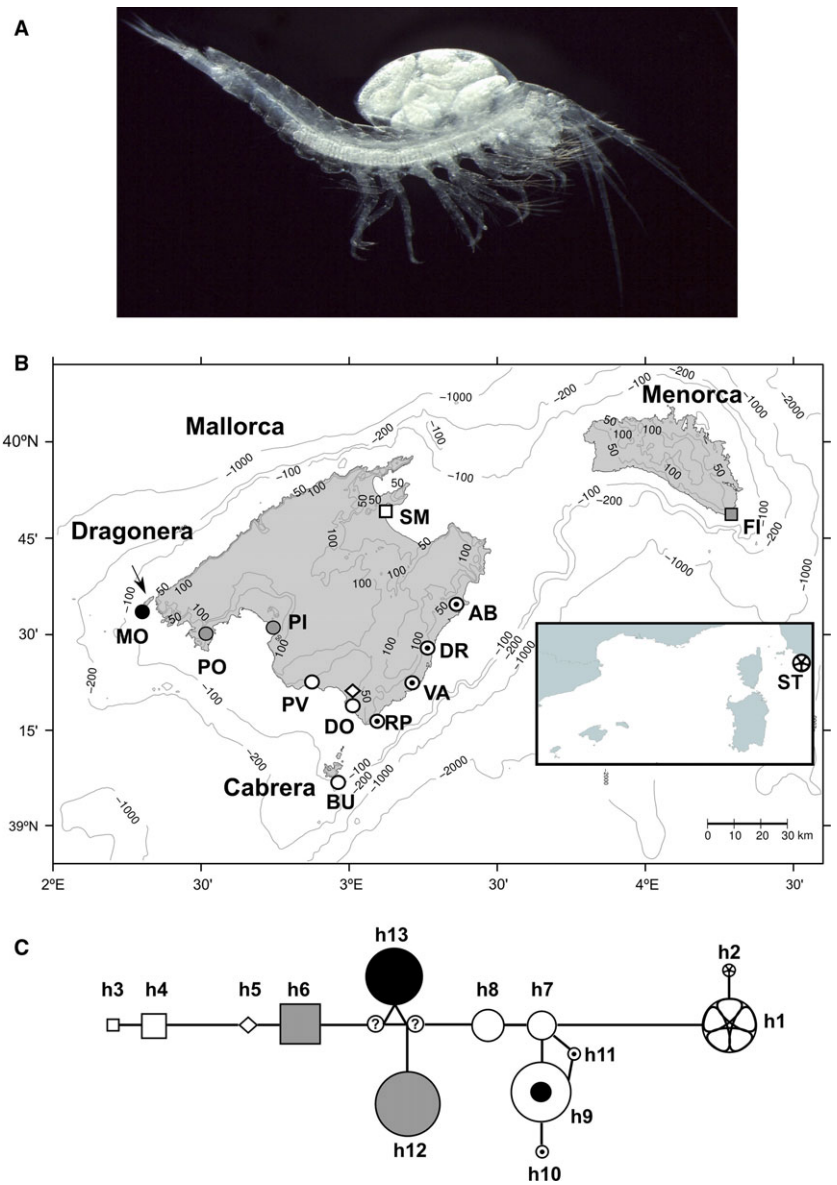

Fig. 1 —A. *Tethysbaena* sp., brooding female from Coves del Drac (Manacor, Mallorca). Notice the dorsal brood pouch. —B. Topographic map indicating sampling sites at the Balearic and Tuscan coasts (see Table 1 for detailed information). Symbols denote molecular entities recorded at each locality (see Figs 2–3). —C. Median-joining network depicting phylogenetic relationships among Balearic *Tethysbaena cox1* mtDNA haplotypes. Circle size proportional to haplotype frequency, with median vectors (i.e. missing intermediates or ancestral haplotypes) indicated by question marks. Branch lengths proportional to number of positions exhibiting nucleotide mutations.

sympatric species), one at Lamkedmyia (Morocco), one at Salalah and another one at Mirbat (both on the Dhofar coast of Oman, Indian Ocean), and an anchialine cave on the SW coast of the Dominican Republic (Hispaniola, Caribbean). Localities and number of specimens studied per site in *T. scabra* and *T. argentarii* are listed in Table 1. Specimens were fixed in 96% ethanol and stored at 4 °C. Genomic DNA was isolated following Wizard® SV 96 Genomic DNA Purification System (Promega) or DNeasy Tissue Kit (Qiagen, Hilden, Germany) manufacturers' protocols. *Cox1* 5′ end sequences were amplified and sequenced as described in Bauzà-Ribot *et al.* (2011) using primer pairs LCO-HCO (Folmer *et al.* 1994; Geller *et al.* 2013). Sequences for the large subunit of nuclear ribosomal DNA (28S rDNA) were obtained for a subset of 84 individuals using the primers 1274-GACCCGTCTT GAAACACGGA (Markmann & Tautz 2005) and D6br-CACACGAAACCCTTCTCCAC (Omilian & Taylor 2001). Chromatograms were edited with CodonCode Aligner v. 5.0.1 (CodonCode Corp., Denham, MA, USA), and DNA sequences aligned with MAFFT v. 7.215 (Katoh & Toh 2008) using default parameters. After alignment, *cox1* and 28S rDNA sequences were 667 bp and 727 bp in length, respectively. The number of parsimony informative sites was 215 for *cox1*, and 54 for 28S rDNA sequences.

Table 1 Localities, number of specimens analysed (*n*) and haplotypes recorded per locality for *T. scabra* and *T. argentarii*. Haplotype (H) and nucleotide (π) diversity are shown with their respective standard deviations in brackets

Code	Site name	Locality	*n*	Haplotype (*n*)	H	π ($\times$ E^{-02})
MO	Cova de sa Font	Dragonera	22	h13 (22)	–	–
PI	Well at es Pil·larí	Palma; Mallorca	21	h12 (21)	–	–
PO	Well at sa Porrassa	Magaluf; Mallorca	8	h12 (8)	–	–
BU	Cova des Burrí	Cabrera	3	h7 (3)	–	–
DO	Cova des Dolç	Ses Salines; Mallorca	8	h8 (6) h5 (2)	0.429 (0.169)	3.838 (1.511)
PV	Cova des Pas de Vallgornera	Llucmajor; Mallorca	4	h7 (3) h8 (1)	0.500 (0.255)	0.746 (0.396)
VA	Cova de Cala Varques C	Manacor; Mallorca	2	h9 (2)	–	–
AB	Cova de s'Abisament	Sant Llorenç; Mallorca	8	h9 (7) h10 (1)	0.250 (0.180)	0.373 (0.269)
RP	Cova des Dracs des Rafal des Porcs	Ses Salines; Mallorca	6	h9 (5) h11 (1)	0.333 (0.215)	0.498 (0.321)
DR	Coves del Drac	Manacor; Mallorca	11	h9 (11)	–	–
SM	Cova de son Sant Martí	Alcúdia; Mallorca	5	h4 (4) h3 (1)	0.400 (0.056)	0.597 (0.354)
FI	Cova de ses Figueres	Sant Lluís; Menorca	10	h6 (10)	–	–
ST	Grotta degli Stretti	Monte Argentario; Tuscany	22	h1 (21) h2 (1)	0.091 (0.081)	0.136 (0.121)

Phylogenetic analyses

Phylogenetic trees were estimated in BEAST v. 1.8.1 (Drummond *et al.* 2012) using a substitution model for the first and second codon sites, and another one for the third positions (see Results). The best substitution model for each partition was selected with pMrAIC v. 1.1 (Nylander 2004). Analyses implementing different coalescence models (see below) were run for 100 million generations sampling every 1000 generations. Convergence of all parameters was assessed using Tracer v. 1.6 (Rambaut *et al.* 2015), whereas consensus tree topologies and posterior node support were estimated with TreeAnnotator (Drummond *et al.* 2012). Maximum Likelihood (ML) analyses were implemented in RAxML v. 7.2.8 (Stamatakis 2006) using a GTR+CAT model with nodal support estimated by 1000 fast bootstrap replicates. Parsimony searches were performed in PAUP v. 4.0b10 with 1000 random addition replicates saving 50 trees per replicate and heuristic searches with tree-bisection-reconnection branch-swapping (Swofford 2002). We performed 10 000 bootstrap replicated to assess clade support.

Species delimitation

We used a generalised mixed Yule coalescent method (GMYC) to determine genetic clusters representing independently evolving entities (Pons *et al.* 2006; Monaghan *et al.* 2009; Fujisawa & Barraclough 2013). The GMYC algorithm compares an ultrametric tree in which all branches are assumed to follow a coalescent diversification model vs. another one based on a mixed Yule coalescent model. The threshold in branching rate at which the transition between these two alternative diversification patterns occurs is computed based on ML. Tree nodes placed below this threshold are considered to correspond to species diversification events, while those above that threshold represent clusters following coalescent processes (Barraclough *et al.* 2003; Fontaneto *et al.* 2007). The independent entities determined by the GMYC model might represent cryptic species. The support of the GMYC-delimited entities is estimated through the comparison of several coalescent and speciation models. The support value of a node is defined as the sum of Akaike weights of candidate delimitation models where the node is included (Fujisawa & Barraclough 2013). The Poisson Tree Processes model (PTP) was alternatively used for species delimitation (Zhang *et al.* 2013). This method models branching events in terms of number of substitutions and does not require the use of ultrametric trees. bPTP also estimates Bayesian support (BS) values for delimited entities on the input tree. A high BS value on a particular node indicates all its descendant nodes, and branches are likely to pertain to a single species. The *cox1* sequence data set used in GMYC and PTP analyses was collapsed to a set of different haplotypes using the Perl script uniqHaplo.pl (Takebayashi 2015).

Population analyses

Number of haplotypes per site, haplotype and nucleotide diversity, and pairwise F_{ST} distances and their significance based on 10 000 permutations were determined using DnaSP v. 5 (Librado & Rozas 2009), Arlequin v. 3.0.1 (Excoffier *et al.* 2005) and MEGA v. 5.2 (Tamura *et al.* 2011). DnaSP analyses excluded all nucleotide positions with missing data, while in MEGA only those positions with missing data in a particular pairwise comparison were excluded. Median-joining network was estimated in Network v. 4.6.1.2 (Bandelt *et al.* 2015). This method builds up minimum spanning trees within a single network, subsequently adding median vectors (consensus sequences) to

reduce tree length. Such vectors can be interpreted as extant unsampled sequences or as extinct ancestral sequences (Bandelt *et al.* 1999). To assess the occurrence of isolation by distance, a Mantel test was performed on genetic and geographic population distances using ZT (Bonnet & Van de Peer 2002). SAMOVA v. 1.0 (Dupanloup *et al.* 2002) was used to identify the geographic groupings that maximised genetic variance between groups of populations. The method calculates F statistics (genetic variance among populations, among populations within groups and among groups; F_{ST}, F_{SC}, F_{CT}, respectively) using AMOVA (Excoffier *et al.* 1992) and identifies the optimum number of population groups for a set of sampled sites taking into account their geographic location. We used 100 simulated annealing processes for each value of *K*.

Estimation of divergence time

C*ox1* divergences in Malacostraca vary broadly, with estimated substitution rates ranging from 1.4 to 2.6% per million years in pairwise comparisons (e.g. Knowlton & Weigt 1998; Finston *et al.* 2007; Kornobis *et al.* 2010). In our case, we decided to use as a proxy the mean substitution rate estimated for another anchialine crustacean, the stygobiont amphipod *Metacrangonyx longipes*, a Balearic endemic that displays a geographic distribution similar to *Tethysbaena*, with some populations even co-occurring in the same caves (Bauzà-Ribot *et al.* 2011, 2012). The *M. longipes cox1* rate (1.32% per lineage and million years, confidence interval 0.89–1.95%) is close to the standard mitochondrial arthropod 2.3% pairwise divergence (1.15% per lineage). This substitution rate was implemented in the *Tethysbaena* data set as an uncorrelated log-normal prior (mean −4.33, standard deviation 0.2) in a relaxed clock in which different coalescence models were explored (e.g. constant population, exponential, expansion or logistic population growth). These models were compared using Bayes factors based on marginal likelihoods estimated by path sampling method. Analyses were performed in BEAST starting from a random tree and using the previously described partition scheme by codon position. The remaining parameters (nucleotide frequencies and substitution model across partitions) and the rate-heterogeneity models were unlinked and estimated from the data set. Search was set to 100 million generations, sampling every 1000. Marginal likelihoods were estimated from a chain of 100 path steps of 1 million generations using a path scheme with a beta Quantile of 0.33 (Baele *et al.* 2012). Node age confidence intervals were estimated in TreeAnnotator after a burnin of the first 10 million generations (Drummond *et al.* 2012).

Results

Phylogenetic analyses and genetic distances

The DNA barcode consisting of a fragment of 667 bp of *cox1* was sequenced from 108 specimens of *T. scabra* (Table 1). *Tethysbaena argentarii* (Italy), *T. atlantomaroccana* (Morocco) and six not yet formally described species from Morocco (3 spp.), Oman (2 spp.) and the Dominican Republic (1 sp.) were used as potential outgroups. Sequences were deposited in European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession numbers LN899288-LN899424. ML, Bayesian and parsimony phylogenetic analyses showed similar tree topologies (Fig. 2 and Fig. S1). *Tethysbaena* of the Balearic Archipelago and the Italian Peninsula conform a single highly supported monophyletic assemblage (with uncorrected genetic p-distances <10%), appearing genetically very distant (>17%) from the remaining congeneric species from other geographical areas (Fig. 2). Phylogenetic relationships within this Balearic + Italy clade were supported unambiguously only between three (or four; see below) of the Balearic lineages, while the positions of *Tethysbaena scabra* from Menorca (FI), *T. argentarii* from Italy, and the populations from a cave in Alcúdia (SM) and one of the two lineages found at cova des Dolç (DO-L1), both on Mallorca, relative to each other, and with respect to the rest of Mallorcan, lineages were not resolved using this DNA sequence data set (Fig. 2). The nuclear 28S rDNA sequences (accession numbers LT159977-LT159999) showed low genetic variation (maximum uncorrected p-distance of 5%) resulting in a poorly resolved phylogenetic tree in which only three clades are supported, namely the sequences of *T. argentarii* from Italy, Alcúdia (SM) and from the remaining samples (Fig. S2).

A species delimitation using *cox1* sequences and GMYC method on the monophyletic Balearic + Italian assemblage revealed the occurrence of a transition in the branching rate from species to the population level 1.1 Myr ago, predicting the presence of eight major lineages (Fig. 3). An ultrametric tree for species delimitation was obtained in BEAST applying a coalescence model and a constant population size prior, two independent substitution models for 1st + 2nd (HKY + G) and third (GTR) codon sites, a relaxed clock with a log-normal prior (mean 1.32%), and excluding 86 identical haplotypes (Fig. 3). The GMYC model was preferred over the null model of coalescent branching rates ($\log L = 88.74$, compared to null model $\log L = 81.31$; $2\Delta L = 14.86$, χ^2 test, d.f. = 3, $P < 0.001$). Similar results were attained applying the Poisson Tree Processes model (PTP) except this analysis collapsed two of the previously deduced *cox1*-based lineages into a single one (as ***e*** in Fig. 3), rendering a total of seven lineages

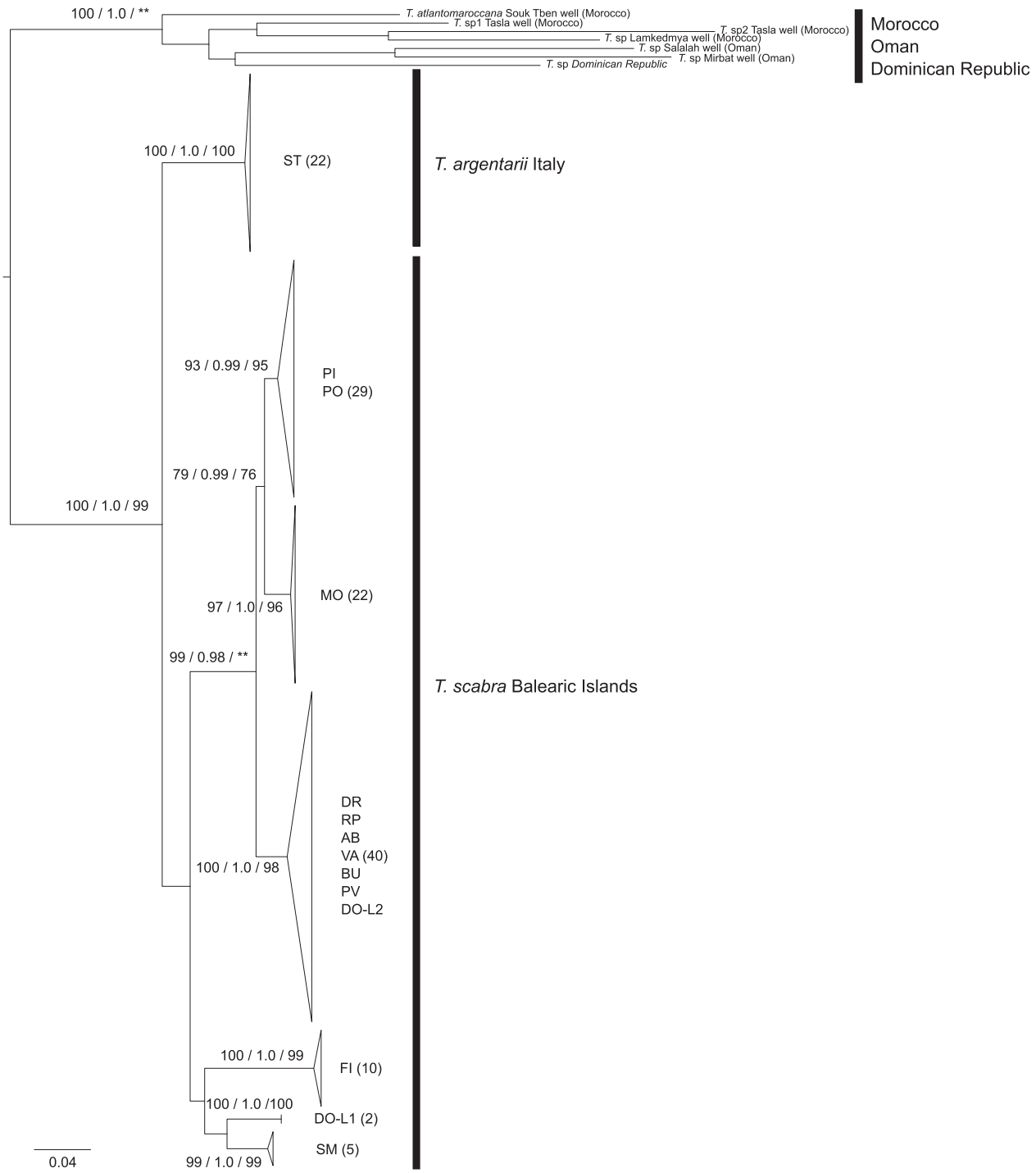

Fig. 2 Maximum likelihood tree based on *cox1* sequences of 108 specimens of *Tethysbaena scabra* from Mallorca, Menorca, Dragonera and Cabrera plus eight *Tethysbaena* species from Italy, Morocco, Oman and the Dominican Republic. Tree rooted by mid-point rooting. Numbers placed on nodes indicate ML bootstrap support (first, ≥70), Bayesian posterior probability (second, ≥0.95) and bootstrap support based on Parsimony criterion (third number, ≥70). Numbers within brackets correspond to number of sequences within each cluster. Locality codes as in Table 1. Note locality DO harbours two divergent lineages (DO-L1 and DO-L2).

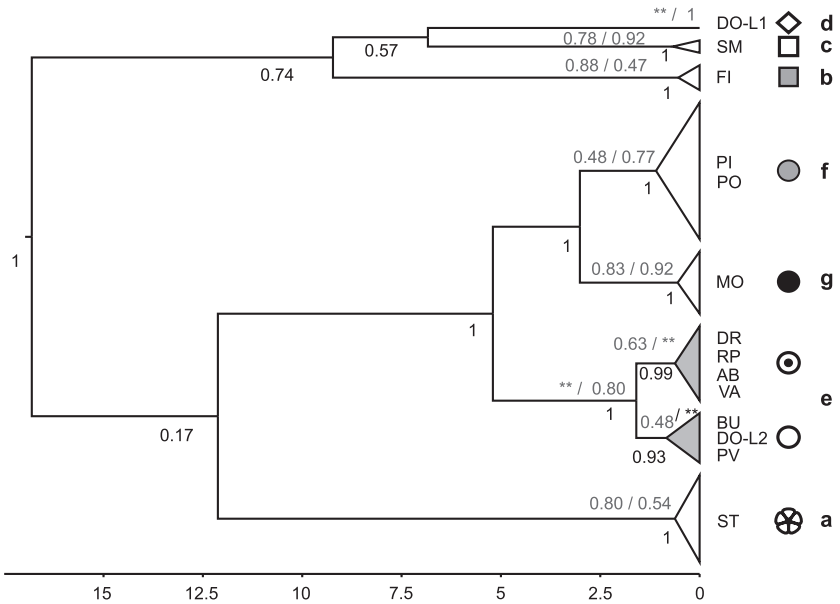

Fig. 3 Molecular entities delimited in a phylogenetic tree based on *cox1* sequences by GMYC and PTP algorithms in W Mediterranean *Tethysbaena* (*T. scabra* and *T. argentarii*). Numbers placed above nodes indicate GMYC (left) and bPTP (right) support values, and numbers below nodes posterior credibility (≥0.95) estimated by Bayesian inference. Asterisks indicate no support. Locality codes as in Table 1 and symbols as in Fig. 1. Single lower case letter codes indicate delimited GMYC and PTP entities (i.e. putative species). Note identical haplotypes were removed from data set before performing analyses.

instead of eight (Fig. 3; see also Fig. 2). One of the lineages (lineage ***a***) corresponds to the formally described species *Tethysbaena argentarii*, which is confined to a single locality on the Italian coast (cave locality ST; Table 1). The remaining lineages are limited to the Balearic Islands: one corresponds to the formally described *T. scabra* from Menorca (lineage ***b***, cave FI, the type locality of the species; Table 1), while the rest occur on Mallorca and two islets placed nearby. Two of them show also a very localised distribution, each in a single cave, one placed on the north coast of the island (lineage ***c***; cave SM; Table 1), the other one on the south coast (lineage ***d***; cave DO; Table 1). The lineage ***d*** displayed a *cox1* sequence highly divergent (lineage DO-L1) from the rest of specimens sampled in the same cave (p-distance = 9.5%; DO-L2), suggesting the occurrence of two sympatric taxa at this locality. The second sympatric lineage ***e*** present in this cave displays a wider geographic distribution covering the south and east coasts of Mallorca plus Cabrera islet (localities DO, DR, RP, AB, VA, BU and PV; Table 1). Finally, two additional related *cox1* lineages (***f*** and ***g***) are apparently confined to the SW of Mallorca (caves PO and PI; Table 1) and to Dragonera islet (cave MO; Table 1), respectively (Fig. 3). The geographic distribution of all these molecular entities is shown in Fig. 1. We did not perform GMYC and PTP analyses based on 28S rDNA sequences due to the low phylogenetic signal displayed by this marker.

Cox1 p-distances among the more divergent Mallorcan lineages average 7.6–9.6% and are on par with their divergence with respect to the formally described species *T. scabra* from Menorca (7.9–8.7%) or *T. argentarii* from Italy (8.3–9.3%). The genetic distance between the latter two species averages 10.2% (Table S1). These divergence values suggest that at least some of the Mallorcan lineages represent cryptic species, in accord with the results of the species delimitation analyses.

Population genetic structure and genetic diversity

Thirteen different *cox1* haplotypes were identified using DnaSP in the populations from the Balearics and Italy (Table 1). Their median-joining network (Fig. 1C) showed a population structure similar to that retrieved in the phylogenetic analysis (Fig. 3). Four haplotypes are shared among several Mallorcan populations. Thus, haplotype h9 appears with a high frequency at VA, AB, RP and DR caves (south/south-east Mallorcan coast), whereas haplotype h7 is present at BU and PV; h8 at DO and PV; and h12 at PI and PO. Haplotype diversity per locality is low or even zero; five caves and two wells displayed each a single haplotype, while the rest of caves harboured a maximum of two haplotypes only. Nucleotide diversity ranged between 0.0014 and 0.0075 except at cave DO, where a remarkably higher value of 0.038 was reached due to the occurrence of two very divergent haplotypes (h8 and h5, each belonging to a different lineage; see above). Mantel test revealed a low significant correlation between genetic and geographic distance in the Balearic localities ($r = 0.603$, $P = 0.01$). In addition, *cox1* pairwise population F_{ST} values were very low or non-significant when comparing localities placed nearby or those sharing haplotypes ($F_{ST} = 0$–0.3), whereas moderate to high in the rest of cases, indicating absence of gene flow ($F_{ST} = 0.5$–1). SAMOVA showed that the optimum number of population

groups necessary to maximise F_{CT} values in the Balearic samples was $K = 6$ ($F_{CT} = 0.886$) ($K = 7$ if *T. argentarii* from Italy is included in the analysis), with a population aggregation pattern equivalent to that resulting from the GMYC and PTP analyses except for the two divergent *cox1* haplotypes present in cave DO; these two haplotypes appear in the same group in the SAMOVA as this analysis groups haplotypes geographically.

Estimation of coalescence times

Coalescence of the 130 *cox1* mitochondrial DNA sequences of *T. scabra* and its closest relative *T. argentarii* was estimated by Bayesian analysis implementing a relaxed molecular clock, as Bayes factors rejected the assumption of a strict clock (see Table 2). A logistic population growth model was preferred over constant population or exponential/expansion population growth models based on Bayes factor values estimated by marginal likelihood using the path sampling method (Table 2). The estimated age for the crown node (time to the most recent common ancestor) of the Balearic + Italy *Tethysbaena* clade varied depending on the population model used between 10.7 Myr (95% higher posterior density, HPD 18.4–4.3 Myr) using the preferred population model to a maximum of 18.1 Myr. Moreover, the estimated node ages for the diversification between and within lineages were relatively similar irrespective of the population model assumed (Table 2). Coalescence of the three lineages present on the south, east and west coast of Mallorca plus Cabrera and Dragonera islets (lineages ***e***, ***f*** and ***g***) was estimated at 3.6 Myr ago under the logistic model (HPD 6.4–1.4 Myr; see Table 2). Ulterior splits presumably took place more recently, *ca*. 2 Myr ago between lineages ***f*** and ***g*** (Palma Bay and Dragonera; HPD 3.4–0.7 Myr), and *ca*. 1.2 Myr ago within lineage ***e*** (between the southern Mallorcan coast + Cabrera and the eastern Mallorcan coast; HPD 2.1–0.4 Myr).

Discussion

Cryptic diversity in Tethysbaena

Some crustacean groups – especially stygobionts – include a remarkable number of cryptic species (Witt & Hebert 2000; Wellborn *et al.* 2005; Leféb ure *et al.* 2006a,b; Witt *et al.* 2006; Finston *et al.* 2007; Seidel *et al.* 2009; Westram *et al.* 2011). For example, Bauzà-Ribot *et al.* (2011) revealed a surprisingly high population genetic structure in the Balearic stygobiont amphipod *Metacrangonyx longipes*, compatible with the genetic divergence expectable among allopatric cryptic sister lineages ('type 1 crypticism'; Trontelj *et al.* 2009). Seemingly, the monophyletic assemblage of *Tethysbaena* thermosbaenaceans studied herein – which includes several highly divergent lineages– could embrace a mosaic of cryptic species. Thus, whereas *T. argentarii* is endemic to a single locality on the Italian coast, six or seven lineages (depending on the analysis performed) occur on the Balearic Islands. Only one of them has been formally described thus far, as *T. scabra*, and is apparently limited to a single cave on Menorca. The rest appear only on Mallorca, and two islets placed nearby. As previously stated, *cox1* genetic distances between all these lineages are relatively high and are in agreement with the results of the species delimitation analyses and SAMOVA presented above. It is remarkable that two Mallorcan lineages (***d*** and ***e***) occur in the same cave (Cova des Dolç, locality code DO; Table 1). They could represent a case of morphological convergence between non-sister lineages after evolution under similar ecological constraints, or due to a lack of morphological differentiation ('type 2 crypticism'; Trontelj *et al.* 2009). This is not the only case of sympatry reported herein because two of the outgroup lineages included in our analyses were collected in the same well in the Moroccan Anti-Atlas.

The 28S rDNA data set, although containing scarce phylogenetic information and with a presumably much larger

Table 2 Marginal Likelihood values estimated by path sampling (ps), and crown node ages (in million years) for the *Tethysbaena* lineages of Fig. 3. Ages were estimated using several coalescence models in BEAST v. 1.8.1 on the 130 *cox1* sequences. A constant population (ct. pop.) size model was assessed under both strict and relaxed uncorrelated log-normal clocks, but the former was rejected by Bayes factors. Hence, analyses assumed a relaxed clock with an uncorrelated log-normal prior. Confidence intervals correspond to logistic growth values

Model	ps	Root	a	b	c	e	f	g	e1	e2	f + g	e + f+g
ct. pop. strict clock	−2393.585	15.458	0.448	0.454	0.62	1.248	0.735	0.41	0.715	Paraphyly	2.679	4.449
ct. pop. log normal	−2388.591	13.287	0.636	0.521	0.685	1.501	0.931	0.578	0.803	0.6	2.424	4.417
Exponential growth	−2382.286	14.581	0.451	0.416	0.596	1.276	0.726	0.42	0.667	0.461	2.352	4.364
Expansion growth	−2389.935	12.654	0.757	0.644	0.788	1.626	1.061	0.706	Paraphyly	0.72	2.501	4.471
Skyride uniform	−2364.180	18.056	0.281	0.245	0.639	1.279	0.470	0.265	0.646	0.243	3.354	6.058
Skyline piecewise ct.	−2374.389	17.996	0.281	0.297	0.643	1.353	0.559	0.301	0.684	0.316	2.290	5.428
Logistic growth	−2316.038	10.663	0.646	0.385	0.523	1.197	0.713	0.43	0.621	0.446	1.964	3.625
Confidence interval												
Lower	na	4.266	0.099	0.042	0.056	0.404	0.202	0.097	0.16	0.112	0.672	1.384
Upper	na	18.393	0.92	0.826	1.127	2.126	1.301	0.821	1.127	0.816	3.42	6.438

coalescence time, supports three of the major lineages detected using mtDNA. More variable nuclear markers would be necessary however to perform a formal species delimitation scrutiny as the GMYC method is known to perform poorly in case of gene trees with many zero length terminal branches (O'Meara 2010). Despite their clear utility in the resolution and discovery of species, coalescent-based species delimitation methods have some limitations (Fujita *et al.* 2012). Ideally, multilocus data should be used to avoid discordances between the genealogy from a particular locus and the history of speciation. In addition, methods that use a fixed tree can overestimate the number of species (Fujita *et al.* 2012). Notwithstanding, the GMYC model has been shown to be useful in cases where divergences are deep, taxon sampling is incomplete or obtaining multilocus data is unattainable (Reid & Carstens 2012).

Phylogeography of anchialine species

The phylogeographic structure of anchialine species seems to be largely determined by natural history traits such as the existence and duration of dispersive larval stages at sea (Santos 2006; Craft *et al.* 2008). Accordingly, a complete lack of population differentiation should be expected in taxa with long-lasting marine planktonic larvae (or shallow population structures, depending on the presence and extent of barriers to subterranean dispersal). Conversely, a strong populational structure and/or species differentiation would be the case in taxa with reduced dispersal capabilities (Santos 2006; Craft *et al.* 2008). The latter should show phylogeographic patterns similar to those exhibited by most groundwater crustaceans, where the reduced dispersal aptitudes and the historical fragmentation of populations have led to distributions often limited to a single island or groundwater system (Trontelj *et al.* 2009). Indeed, this is the condition approached in the Hawaiian anchialine shrimp *Halocaridina rubra* Holthuis, 1963 (Santos 2006; Craft *et al.* 2008). The remarkable level of populational genetic structure shown by this shrimp throughout the Archipelago seems to be the result of allopatric fragmentation and isolation by distance, coupled with the occurrence of episodes of short-range coastal dispersal. *Halocaridina* has life history traits such as large eggs, lecithotrophic larvae and abbreviated larval development that make long-distance dispersal very unlikely (Santos 2006; Craft *et al.* 2008).

However, and as stated above, other anchialine taxa show no trace of genetic differentiation. This is the case of the Philippine anchialine neritilid gastropod *Neritilia cavernicola* Kano & Kase, 2004; where the combination of amphidromy and drifting of planktonic larvae would explain the lack of differentiation between populations placed 200 km apart (Kano & Kase 2004). The same holds for the anchialine alpheid shrimp *Metabetaeus lohena* Banner & Banner, 1960, that appears genetically undifferentiated throughout the islands of the Hawaiian Archipelago (Russ *et al.* 2010).

In accord with Santos (2006) and Craft *et al.* (2008), we show that the non-diadromous, strictly anchialine *Tethysbaena* thermosbaenaceans from the Balearics and Italy display a clear-cut phylogeographic pattern. Six of the seven *cox1* lineages recognised within this monophyletic assemblage appear in allopatry, the only exception being lineage ***d***, which shows an extremely localised distribution limited to a single cave placed within the geographic range of lineage ***e***. All lineages show a distribution reduced to a single cave (***a***–***d***, ***g***) or to short strips of coast (***e***, ***f***) not exceeding 60 km in length. Therefore, the anchialine belt developed around the islands is not as continuous as might be expected and allows the maintenance of discrete populations of strictly anchialine species that might have been thought initially that showed no differentiation.

Sea-level oscillations and historical biogeographic patterns in anchialine thermosbaenaceans

The islands and islets of the Balearic Archipelago harbouring *Tethysbaena* (Mallorca, Menorca, Cabrera and Dragonera) are located on a common submarine promontory and have remained isolated from the continent and from the rest of Balearic Islands (Ibiza and Formentera) since the beginning of the Pliocene, when the Mediterranean was re-flooded after the Messinian salinity crisis (5.33 Myr ago; Krijgsman *et al.* 1999). With the advent of the Pleistocene 2.59 Myr ago, broad glacio-eustatic oscillations have modified repeatedly the size and shape of the emerged lands, leading to their recurrent connection and separation. Thus, Mallorca and Menorca were split off and had most of their lowlands submersed about 780 000 years ago, when sea level reached ca. +90 m above its present stand in the Balearic area (Cuerda 1975). Conversely, they conformed a single composite island as recently as at the Last Glacial Maximum (21 000 years ago), when sea level dropped to ca. −134 m (Cuerda 1975; Lambeck *et al.* 2014).

Our coalescence estimations based on *cox1* sequences (Table 2) suggest that presumably at the Early Tortonian (10.7 Myr ago; with the caveat of the broad HPD associated with this dating: 18.4–4.3 Myr) an ancestral Balearic + Italy *Tethysbaena* monophyletic assemblage split into five different lineages (***d***, ***c***, ***b***, [***e*** + ***f*** + ***g***], and ***a***; see Fig. 3). In any case, this time span largely precedes the onset of the broad glacio-eustatic oscillations of the Quaternary and indicates that the formation of the major lineages within this species assemblage responded to other causes. Descendants of these five lineages currently persist on Italy (***a***), Menorca (***b***), the north coast of Mallorca (***c***), the south coast of Mallorca (***d***), and the south and

south-east coasts of Mallorca plus Dragonera and Cabrera islets ([***e*** + ***f*** + ***g***]).

Only the diversification that took place within the latter lineage [***e*** + ***f*** + ***g***] could be coeval with the glacio-eustatic oscillations of the Quaternary. Indeed, the estimated datings for the splits affecting this lineage fall, or partially overlap, into the Pleistocene. Thus, the coalescence of Mallorcan lineages ***e*** and [***f*** + ***g***] has been estimated at 3.6 Myr ago (HPD 6.4–1.4 Myr). Other splits would have subsequently followed, as those between lineages ***f*** and ***g*** about 2.0 Myr ago (HPD 3.4–0.7 Myr), or within lineage ***e*** 1.2 Myr ago (HPD 2.1–0.4 Myr). The broad confidence intervals associated with these datings–which partially overlap– impede to associate the corresponding splits to any particular transgressive–regressive sea-level cycle. In any event, the topography of Fig. 1B shows that Lower Pleistocene transgressive phases such as those recorded at +55 m a.s.l. (530 000 years ago) or at +90 m a.s.l. (780 000 years ago) on the south coast of Mallorca (see Cuerda 1975: 49; 81) could be in the origin of lineages ***f*** and ***g*** or have caused the split within lineage ***e***, in a way similar to that described by Notenboom (1991; i.e. vicariance triggered by marine regressions) to explain the diversification of other stygobiont crustaceans of direct marine derivation. This mechanism has been suggested elsewhere to explain the divergence of *Metacrangonyx* lineages in the same area (Bauzà-Ribot *et al.* 2011, 2012). The impact on population genetic structure of more recent eustatic oscillations (for instance, the maximum lowstand at the Last Glacial Maximum) cannot be easily detected with the genetic marker at hand, and a much faster evolving sequence should be used for this purpose.

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References

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M. A. & Alekseyenko, A. V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology Evolution*, *29*, 2157–2167.

Bandelt, H. J., Forster, P. & Röhl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, *16*, 37–48.

Bandelt, H. J., Foster, P. & Röhl, A. (2015) *Network v. 4.6.1.3*. Available via http://www.fluxus-engineering.com/sharenet.htm.

Barraclough, T. G., Birky, C. W. & Burt, A. (2003). Diversification in sexual and asexual organisms. *Evolution*, *57*, 2166–2172.

Bauzà-Ribot, M. M., Jaume, D., Fornòs, J. J., Juan, C. & Pons, J. (2011). Islands beneath islands: phylogeography of a groundwater amphipod crustacean in the Balearic archipelago. *BMC Evolutionary Biology*, *11*, 221.

Bauzà-Ribot, M. M., Juan, C., Nardi, F., Oromí, P., Pons, J. & Jaume, D. (2012). Mitogenomic phylogenetic analysis supports continental-scale vicariance in subterranean thalassoid crustaceans. *Current Biology*, *22*, 2069–2074.

Bishop, R. E., Humphreys, W. F., Cukrov, N., Zic, V., Boxshall, G. A., Cukrov, M., Iliffe, T. M., Kršinić, F., Moore, W. S., Pohlman, J. W. & Sket, B. (2015). Anchialine" redefined as a subterranean estuary in a crevicular or cavernous geological setting. *Journal of Crustacean Biology*, *35*, 511–514.

Bonnet, E. & Van de Peer, Y. (2002). Zt: a software tool for simple and partial Mantel tests. *Journal of Statistical Software*, *7*, 1–12.

Craft, J. D., Russ, A. D., Yamamoto, M. N., Iwai., T. Y., Jr, Hau, S., Kahiapo, J., Chong, C. T., Ziegler-Chong, S., Muir, C., Fujita, Y., Polhemus, D. A., Kinzie, R. A., III & Santos, S. R. (2008). Islands under islands: The phylogeography and evolution of *Halocaridina rubra* Holthuis, 1963 (Crustacean: Decapoda: Atyidae) in the Hawaiian archipelago. *Limnology & Oceanography*, *53*, 675–689.

Cuerda, J. (1975). *Los Tiempos Cuaternarios en Baleares*. Palma de Mallorca: Diputación Provincial de Baleares.

Drummond, A. J., Suchard, M. A., Xie, D. & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology Evolution 29*, 1969–1973.

Dupanloup, I., Schneider, S. & Excoffier, L. (2002). A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, *11*, 2571–2581.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, *131*, 479–491.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, *1*, 47–50.

Finston, T. L., Johnson, M. S., Humphreys, W. F., Eberhard, S. M. & Halse, S. A. (2007). Cryptic speciation in two widespread subterranean amphipod genera reflects historical drainage patterns in an ancient landscape. *Molecular Ecology*, *16*, 355–365.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). NA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–299.

Fontaneto, D., Herniou, E. A., Boschetti, C., Caprioli, M., Melone, G., Ricci, C. & Barraclough, T. G. (2007). Independently evolving species in asexual bdelloid rotifers. *PLoS Biology*, *5*, e87.

Fujisawa, T. & Barraclough, T. G. (2013). Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent (GMYC) approach: a revised method and evaluation on simulated datasets. *Systematic Biology*, *62*, 707–724.

Fujita, M. K., Leaché, A. D., Burbrink, F. T., McGuire, J. A. & Moritz, C. (2012). Coalescent-based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution*, *27*, 480–488.

Geller, J., Meyer, C., Parker, M. & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, *13*, 851–861.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & De Waard, J. R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, *270*, 313–321.

Hebert, P. D. N., Ratnasingham, S. & De Waard, J. R. (2003b). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society B: Biological Sciences*, *270*(Suppl. 1), S96–S99.

Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *359*, 183–195.

Iliffe, T. M. & Kornicker, L. S. (2009). Worldwide diving discoveries of living fossil animals from the depths of anchialine and marine caves. *Smithsonian Contributions to the Marine Sciences*, *38*, 269–280.

Jaume, D. (1993). Fauna carcinològica de les aigües continentals. *Història Natural de l'Arxipèlag de Cabrera*. In: J. A. Alcover, E. Ballesteros & J. J. Fornós (Eds.) (pp. 309–322). Palma de Mallorca: Ed. Moll/CSIC.

Jaume, D. (2008). Global diversity of spelaeogriphaceans & thermosbaenaceans (Crustacea; Spelaeogriphacea & Thermosbaenacea) in freshwater. *Hydrobiologia*, *595*, 219–224.

Kano, Y. & Kase, T. (2004). Genetic exchange between anchialine cave populations by means of larval dispersal: the case of a new gastropod species *Neritilia cavernicola*. *Zoologica Scripta*, *33*, 423–437.

Katoh, K. & Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics*, *9*, 286–298.

Knowlton, N. & Weigt, L. A. (1998). New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society B: Biological Sciences*, *265*, 2257–2263.

Kornobis, E., Pálsson, S., Kristjánsson, B. K. & Svavarsson, J. (2010). Molecular evidence of the survival of subterranean amphipods (Arthropoda) during Ice Age underneath glaciers in Iceland. *Molecular Ecology*, *19*, 2516–2530.

Krijgsman, W., Hilgen, F. J., Raffi, I., Sierro, F. J. & Wilson, D. S. (1999). Chronology, causes and progression of the Messinian salinity crisis. *Nature*, *400*, 652–655.

Lambeck, K., Rouby, H., Purcell, A., Sun, Y. & Sambridge, M. (2014). Sea level and global ice volumes from the Last Glacial Maximum to the Holocene. *Proceedings of the National Academy of Sciences*, *111*, 15296–15303.

Lefébure, T., Douady, C. J., Gouy, M. & Gibert, J. (2006a). 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.

Lefébure, T., Douady, C. J., Gouy, M., Trontelj, P., Briolay, J. & Gibert, J. (2006b). Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution in extreme environments. *Molecular Ecology*, *15*, 1797–1806.

Librado, P. & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*, 1451–1452.

Markmann, M. & Tautz, D. (2005). Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*, 1917–1924.

Molins, A., Mayol, M. & Rosselló, J. A. (2009). Phylogeographical structure in the coastal species *Senecio rodriguezii* (Asteraceae), a narrowly distributed endemic Mediterranean plant. *Journal of Biogeography*, *36*, 1372–1383.

Monaghan, M. T., Wild, R., Elliot, M., Fujisawa, T., Balke, M., Inward, D. J., Lees, D. C., Ranaivosolo, R., Eggleton, P., Barraclough, T. G. & Vogler, A. P. (2009). Accelerated species inventory on Madagascar using coalescent-based models of species delineation. *Systematic Biology*, *58*, 298–311.

Notenboom, J. (1991). Marine regressions and the evolution of groundwater dwelling amphipods (Crustacea). *Journal of Biogeography*, *18*, 437–454.

Nylander, J. A. A. (2004). *pMrAIC.pl version 1.1. (computer program and manual)*. Distributed by Author, Evolutionary Biology Centre, Uppsala University.

O'Meara, B. C. (2010). New heuristic methods for joint species delimitation and species tree inference. *Systematic Biology*, *59*, 59–73.

Omilian, A. R. & Taylor, D. J. (2001). Rate acceleration and long-branch attraction in a conserved gene of cryptic daphniid (Crustacea) species. *Molecular Biology and Evolution*, *18*, 2201–2212.

Pomar, L. (1979). La evolución tectonosedimentaria de las Baleares: análisis critico. *Acta Geològica Hispànica*, *14*, 293–310.

Pons, J., Barraclough, T. G., Gómez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., Kamoun, S., Sumlin, W. D. & Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, *55*, 595–609.

Pretus, J. L. (1991) *Estudio Taxonómico, Biogeográfico y Ecológico de los Crustáceos epigeos e hipogeos de las Baleares (Branchiopoda, Copepoda, Mystacocarida y Malacostraca)*. Ph. D. Thesis, Universitat de Barcelona.

Rambaut, A., Suchard, M. A., Xie, D. & Drummond, A. J. (2015) *Tracer v1.6*. Available via http://beast.bio.ed.ac.uk/Tracer.

Reid, N. M. & Carstens, B. C. (2012). Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evolutionary Biology*, *12*, 196.

Russ, A. D., Santos, S. R. & Muir, C. (2010). Genetic population structure of an anchialine shrimp, *Metabetaeus lohena* (Crustacea: Alpheidae), in the Hawaiian Islands. *Revista de Biología Tropical*, *58*, 159–170.

Santos, S. R. (2006). Patterns of genetic connectivity among anchialine habitats: a case study of the endemic Hawaiian shrimp *Halocaridina rubra* on the island of Hawaii. *Molecular Ecology*, *15*, 2699–2718.

Seidel, R. A., Brian, K., Lang, B. K. & Berg, D. J. (2009). Phylogeographic analysis reveals multiple cryptic species of amphipods (Crustacea: Amphipoda) in Chihuahuan Desert springs. *Biological Conservation*, *142*, 2303–2313.

Shimomura, M. & Fujita, Y. (2009). First record of the thermosbaenacean genus *Halosbaena* from Asia: *H. daitoensis* sp. nov. (Peracarida: Thermosbaenacea: Halosbaenidae) from an anchialine cave of Minamidaito-jima Is., in Okinawa, southern Japan. *Zootaxa*, *1990*, 55–64.

Stamatakis, A. (2006). AxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, *22*, 2688–2690.

Stock, J. H., Iliffe, T. M. & Williams, D. (1986). The concept "anchialine" reconsidered. *Stygologia*, *2*, 90–92.

Swofford, D. (2002). *PAUP*: Phylogenetic Analysis using Parsimony* (and other methods)*, *4.0b10*. Sunderland, MA: Sinauer Associates.

Takebayashi, N. (2015). *uniqHaplo.pl*. Available via http://raven.iab.alaska.edu/~ntakebay/teaching/programming/perl-scripts/perl-scripts.html.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731–2739.

Trontelj, P., Douady, C. J., Fišer, C., Gibert, J., Gorićki, Š., Lefébure, T., Sket, B. & Zakšek, V. (2009). A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts?. *Freshwater Biology*, *54*, 727–744.

Wagner, H. P. (1994). A monographic review of the Thermosbaenacea (Crustacea: Peracarida). *Zoologische Verhandelingen*, *291*, 1–338.

Wagner, H. P. (2012). *Tethysbaena ophelicola* n. sp. (Thermosbaenacea), a new prime consumer in the Ophel biome of the Ayyalon Cave, Israel. *Crustaceana*, *85*, 1571–1587.

Wellborn, G. A., Cothran, R. & Bartholf, S. (2005). Life history and allozyme diversification in regional ecomorphs of the *Hyalella azteca* (Crustacea: Amphipoda) species complex. *Biological Journal of the Linnean Society*, *84*, 161–175.

Westram, A. J., Jokela, J., Baumgartner, C. & Keller, I. (2011). Spatial distribution of cryptic species diversity in European freshwater amphipods (*Gammarus fossarum*) as revealed by pyrosequencing. *PLoS One*, *6*, e23879.

Witt, J. D. S. & Hebert, P. D. N. (2000). Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. *Canadian Journal of Fisheries and Aquatic Sciences*, *57*, 687–698.

Witt, J. D. S., Threloff, D. L. & Hebert, P. D. N. (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, *15*, 3073–3082.

Zhang, J., Kapli, P., Pavlidis, P. & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, *29*, 2869–2876.

Supporting Information

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

Fig. S1. Strict consensus cladogram build from 49 600 parsimony trees of 698 steps (left panel) and Bayesian tree topology (right panel) based on *cox1* sequences of 108 specimens of *Tethysbaena scabra* from Mallorca, Menorca, Dragonera and Cabrera plus eight *Tethysbaena* species from Italy, Morocco, Oman and the Dominican Republic.

Fig. S2. Strict consensus cladogram build from 357 parsimony trees (107 steps) based on 28S rDNA sequences of 84 specimens of *Tethysbaena scabra* from Balearic Islands and *T. argentarii* from Italy.

Table S1. Pairwise distances between collecting sites.