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Phylogeography and phylogeny of the genus *Acanthonyx* (Decapoda, Epialtidae) in the north-east Atlantic and Mediterranean

Ana I. Tavares, M. Pilar Cabezas, Raquel Xavier, Madalena Branco, Fernando P. Lima, Rui Seabra, Pedro A. Ribeiro, Evandro P. Lopes & António M. Santos

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The genus *Acanthonyx* Latreille, 1828 (Majoidea, Epialtidae) contains 17 or 18 known species, depending on competing taxonomic views, that are widely distributed across the world. Morphologically, most species look superficially alike, and therefore, similar taxonomic concepts have been described under different names. Consequently, there is today a considerable list of synonyms, which further complicates the taxonomy of the genus *Acanthonyx*. In this study, we conducted a phylogeographical and phylogenetic analysis of populations of the genus *Acanthonyx* in the NE Atlantic, Mediterranean and Macaronesia using the mitochondrial cytochrome oxidase subunit I (COI) and the nuclear 28S rRNA loci. Our phylogenetic and phylogeographical results revealed that *Acanthonyx lunulatus sensu lato* is a complex of three distinct lineages: one corresponding to the previously described *Acanthonyx brevifrons*, another to *A. lunulatus sensu stricto* and a third to a yet undescribed group. Whereas our results confirms that *A. brevifrons* deserves the status of a species, as it can be easily distinguishable from *A. lunulatus* by a few morphological traits, we could not find any such traits suitable for the discrimination between *A. lunulatus sensu stricto* and the third lineage. Furthermore, the degree of COI divergence between this lineage and *A. lunulatus* is below average levels for Decapoda species. Yet, no shared haplotypes have been detected between them. The differences found in the nuclear gene (indels), together with the sympatric occurrence of the two forms, prompt for a more detailed analysis of this group. Overall, the results show that significant genetic differentiation between specimens with similar morphology occurs in the Epialtidae, thus reinforcing the importance of combining morphological and genetic tools to fully resolve the taxonomy of these decapods.

Corresponding author: *António. M. Santos, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal; UniCV, Universidade de Cabo Verde, Cabo Verde and Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal.*
E-mail: amsantos@fc.up.pt.
Ana I. Tavares, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal and Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal. E-mail: ana.isa_@hotmail.com
M. Pilar Cabezas, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal and UniCV, Universidade de Cabo Verde, Cabo Verde. E-mail: pilarcabezas@cibio.up.pt
Raquel Xavier, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal, UniCV, Universidade de Cabo Verde, Cabo Verde and School of Biological Sciences, Cardiff University, Cardiff, CF10 3AX UK. E-mail: raq.xavier@cibio.up.pt
Madalena Branco, Fernando P. Lima, and Rui Seabra, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal and

UniCV, Universidade de Cabo Verde, Cabo Verde. E-mails: madalenasbranco@gmail.com, fplima@gmail.com, ruisea@gmail.com
Pedro A. Ribeiro, MARE – Marine and Environmental Sciences Centre & IMAR – Institute of Marine and Okeanos- R&D Center, University of the Azores, 9901-862 Horta, Portugal and Okeanos – R&D Center, University of the Azores, 9901-862 Horta, Portugal. E-mail: pribeiro@uac.pt
Evandro P. Lopes, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal and Faculty of Engineering and Marine Sciences, Cabo Verde University, Mindelo, São Vicente, Cabo Verde. E-mail: evandrobiologia007@gmail.com
António M. Santos, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal and Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal and UniCV, Universidade de Cabo Verde, Cabo Verde. E-mail: amsantos@fc.up.pt

Introduction

Among true crabs (Decapoda, Brachyura), the superfamily Majoidea Samouelle, 1819 (which includes the commonly known spider crabs) is one of the most diversified and is thought to contain more than 800 species (Rice 1988). Historically, it has been a problematic group, undermined by several ambiguities at the level of family/subfamily definitions and their hierarchical placement. The recent work of Ng *et al.* (2008) on the extant brachyurans was a step forward in the reorganization of the Majoidea, not only because it contributed to settling many pending taxonomic or nomenclatural issues, but also because it clearly pinpointed those in need of further revision. Although the monophyly of the Majoidea is consensual (Hultgren & Stachowicz 2008), the incorporation of molecular and larval morphology data into phylogenetic reconstructions showed that the internal placement of some families and genera is still far from being resolved (Marques & Pohle 2003; Hultgren & Stachowicz 2008; Mahon & Neigel 2008; Hultgren *et al.* 2009).

Within Majoidea, the family Epialtidae MacLeay, 1838 (*sensu* Ng *et al.* 2008), is the most species rich, with around 380 species, and probably one of the most morphologically heterogeneous (Colavite *et al.* 2014). Given the conspicuous nature of this group, more than half of its species were described before the 1900s and placed into various genera, contributing substantially to the current intricate list of synonyms (Ng *et al.* 2008). Adding to this complexity, the pace at which new species and genera are being described is quite high (e.g. Forges & Ng 2009; Tavares & Santana 2011; Ng & De Forges 2013, 2015). Despite these recent advancements, it is surprising that very few molecular studies have addressed the phylogenetic relationships of Epialtidae below the genus level (see Gomes 2013). This is also true for the Majoidea as a whole, where only *Mithrax* Desmarest, 1823, *Mithraculus* White, 1847, and *Maja* Lamarck, 1801, were subject of phylogenetic analysis (Baeza *et al.* 2009; Sotelo *et al.* 2009; Windsor & Felder 2009). Presently, the NCBI Taxonomy Browser (Benson *et al.* 2009) lists only 28 epialtid species (plus two environmental samples) for which nucleotide data exist, whereas the BOLD System V3 (Ratnasingham & Hebert 2007) comprises 46 barcodes. Yet, most of these data result from the suprageneric analysis (Hultgren *et al.* 2009) and not from research targeted on specific genera.

Acanthonyx Latreille, 1828, comprises 17–18 species (Emparanza *et al.* 2007; Ng *et al.* 2008) distinguished mainly by the shape of the carapace, its ornamentation and the shape of rostral teeth. The morphology of most species is superficially similar, and therefore, similar taxonomic concepts have been described under different names. As a consequence, synonyms abound, further complicating the taxonomy of this genus. A comprehensive revision of *Acanthonyx* is missing and identification keys cover only non-overlapping, albeit large, regions such as the eastern Atlantic (Manning & Holthuis 1981) and the western Indo-Pacific (Griffin & Tranter 1986). Furthermore, a global treatment of American species is still absent, despite some recent taxonomic and nomenclatural rearrangements and evidence of possible cryptic lineages in some widely distributed species (Emparanza *et al.* 2007).

Acanthonyx lunulatus Risso (1816) is the only species of the genus *Acanthonyx* known to occur in the Mediterranean and the NE Atlantic region, extending southwards down to Namibia, reaching the Kunene River (Manning & Holthuis 1981). *A. lunulatus* occurs in shallow waters, inhabiting rocky substrates and usually found attached to algae (Sanz-Brau 1989). Such widespread distribution suggests a high capacity for dispersion. Like most brachyurans, dispersion in Epialtidae is probably driven

by larval stages, although rafting in seaweed cannot be excluded, as it was already reported for a few species, such as those from the genera *Hyas* Leach, 1814, and *Macropodia* Leach, 1814 (Thiel & Gutow 2005), and *Libinia dubia* H. Milne Edwards, 1834 (Williams 1984). The development of *A. lunulatus* includes two zoeae and one megalopa stages, thus following the typical pattern in the Majoidea (Guerao & Abelló 1996; Kornienko & Korn 2007). However, larval duration for *A. lunulatus* is not completely known: the two zoeae stages can endure a maximum of 15 days (Guerao & Abelló 1996), but total planktonic larval duration (PLD) might be longer depending on the duration of the megalopa. PLD of small Majoidea typically varies between 15 and 25 days (e.g. Santana *et al.* 2004a,b; Kornienko & Korn 2007; Santana & Marques 2009; Colavite *et al.* 2014), but can extend up to 120 days (Kelly & Palumbi 2010) hinting at low levels of genetic structure. Other Atlantic–Mediterranean invertebrate species with comparable larval duration have revealed considerable patterns of genetic differentiation (Sá-Pinto *et al.* 2008; Zulliger *et al.* 2009) and even cryptic species (Sá-Pinto *et al.* 2005). Although *A. lunulatus* could potentially display such differentiation patterns, available genetic data are scarce as it has been mostly used as an outgroup in phylogenetic analysis of other majoid genera (Windsor & Felder 2014) or higher taxa (Matzen da Silva *et al.* 2011).

The taxonomy of *A. lunulatus* has also been far from stable. It was originally described by Risso (1816) as *Maia lunulatus* from Nice (France), but was later moved into the genus *Acanthonyx*. More or less concomitantly, several species were independently described under different genera (e.g. *Acanthonyx viridis* Costa, 1838, *Gonosoma viridis* Costa, 1844, *Maia glabra* Latreille, 1836) which are currently considered synonymous of *A. lunulatus*. From the several synonymized names, *Inachus levigatus* Rafinesque, 1814, is a special case, because the name has priority over *lunulatus* from Risso (1816), but given that the latter has become firmly established in carcinological literature, the former has been suppressed (ICZN, 1959). Another unresolved case is *A. brevifrons* Milne-Edwards, 1869; which was described from Cape Verde islands and, according to its author, also occurred in the Azores. Given its superficial similarity with *A. lunulatus*, Zariquiey Alvarez (1968) deemed *A. brevifrons* as a synonym of the former. This taxonomic view was supported by several authors, either in the late 1800s (Miers, 1886) or more recently (Emparanza *et al.* 2007; Ng *et al.* 2008), but according to others the two species should be considered as distinct (Manning & Holthuis 1981).

The aim of the present work was to use a molecular approach to elucidate many of the above-mentioned problems which stem from poor diagnosis or descriptions, combined with morphological similarities between *Acanthonyx* forms. To do so, we sequenced portions of one mitochondrial gene, the cytochrome c oxidase subunit I, and one nuclear gene, 28S ribosomal RNA gene, to reconstruct the phylogenetic relations within *A. lunulatus sensu lato* from the north-east Atlantic, the Mediterranean and Macaronesia regions.

Materials and methods

Sample collection and taxonomic identification

A total of 208 individuals were collected along the Mediterranean and the north-east Atlantic coasts, from the Aegean Sea to the Alboran Sea, and from southern Spain down to Morocco, respectively. Samples were also obtained from the Macaronesian archipelagos of the Azores, Selvagens and Canaries. Sampling was conducted between 2007 and 2014 and included a total of 41 sampling sites (Fig. 1; Table S1). Specimens were collected among intertidal algae and preserved *in situ* in 96% ethanol. In the laboratory, all specimens were observed under a standard binocular microscope and, at first, identified through characteristics such as the shape of the carapace and lateral lobes, following the diagnosis of Zariquiey Alvarez (1968) which considers a single species (*A. lunulatus*) for the whole Mediterranean and NE Atlantic, including the Macaronesia. Once we realized that the specimens from Cape Verde differed consistently in a single trait (the number of lateral lobes in the carapace), we switched to the diagnosis of Manning & Holthuis (1981), which distinguishes *A. lunulatus* from *A. brevifrons* and includes two additional eastern Atlantic species only known from the Gulf of Guinea. According to these authors, *A. brevifrons* is thought to be restricted to Cape Verde and the Azores.

DNA extraction, polymerase chain reaction and gene amplification

Genomic DNA was extracted from muscle tissue of legs using the commercial kit Jetquick (GenoMed). DNA amplification was achieved through polymerase chain reaction (PCR) for one mitochondrial fragment – cytochrome c oxidase subunit 1 (COI) – and one nuclear fragment – 28S ribosomal RNA. Primers used for COI amplification were jgLCO1490 and jgHCO2198 (Geller *et al.* 2013), and for the 28S ribosomal RNA gene, we used the primers designed by Hultgren & Stachowicz (2008). The two genes, COI and 28S, were amplified for 197 and 28 *Acanthonyx* individuals, respectively.

For the COI, PCR conditions were: initial denaturation for 4 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 50 s at 45 °C and 1 min at 72 °C; a final extension was performed for 10 min at 72 °C. For the amplification of

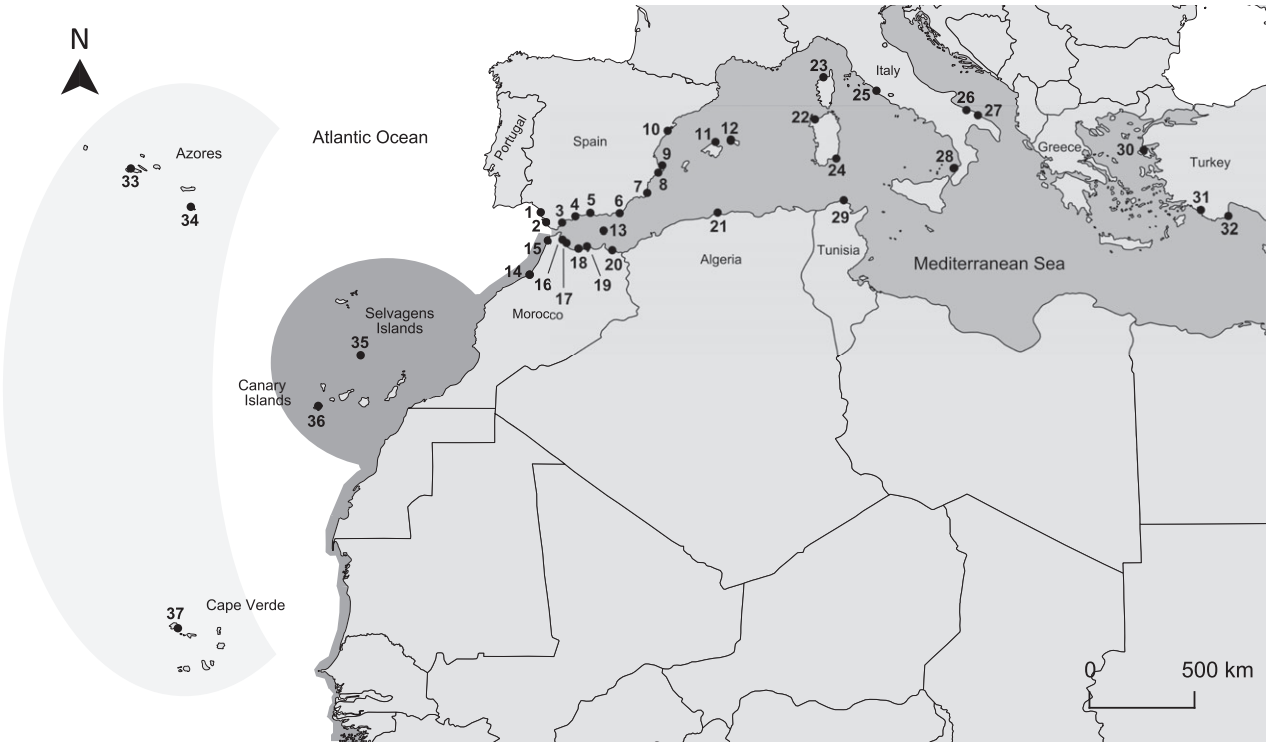

Fig. 1 Map of sampling localities included in the present work: 1. Chipiona; 2. Conil; 3. Torreguadiaro; 4. Benalmadena; 5. Herradura; 6. Cabo da Gata; 7. Cabo de Palos; 8. Cala del Tío Ximo; 9. Dénia; 10. Peniscola; 11. Cala de Sant Vicenç; 12. Cala Blanca and Cap d'Artrutx; 13. Alboran Island; 14. Temara and Le Falouque; 15. Asilah; 16. Azla; 17. Zaouia; 18. Dos Torres and Cala Iris; 19. Al Hoceima; 20. Raselma Nador; 21. Algiers; 22. Porto Torres; 23. L'Île-Rousse; 24. Simius; 25. Santa Marinella; 26. Giovinazzo; 27. Monopoli; 28. Formiggi beach and Tropea; 29. Bizerte; 30. Molivos; 31. Fethiye; 32. Çirali Limani; 33. Faial; 34. Santa Maria; 35. Selvagens; 36. El Hierro; 37. Mindelo. Distribution of *Acanthonyx lunulatus sensu stricto* in dark grey and *A. brevifrons* in light grey.

28S, the PCR conditions were as follows: an initial denaturation for 4 min at 94 °C, followed by 40 cycles of 50 s at 45 °C, 1 min at 65 °C and 90 s at 72 °C; a final extension was performed at 72 °C for 10 min. The Platinum™ Taq (Invitrogen, Paisley, UK) was used and the total volume of reactions was of 25 μL. Amplification products were sent to Beckman Coulter Genomics (UK) for purification and Sanger sequencing. The nuclear gene was sequenced in both directions to ensure identification of heterozygotes. Sequences were blasted to the NCBI database on GenBank to confirm the species identification and were thereafter deposited in GenBank (see Table S1). Chromatograms were checked and sequences were aligned for posterior phylogenetic analysis using CODONCODE ALIGNER 4.2.4 (CodonCode, Dedham, MA, USA).

Estimates of genetic diversity

The amino acid translation was examined for all sequences of COI to ensure that no gaps or premature stop codons were present in the alignment using MEGA version 6 (Tamura *et al.* 2013). Two measures of mtDNA diversity were estimated for each locality, using ARLEQUIN version 3.5.1.2 (Excoffier & Lischer 2010): haplotype diversity (Hd) and nucleotide diversity (π). Tajima's *D* and Fu's FS neutrality tests were also estimated. Localities with less than four individuals or that displayed no polymorphism were omitted from the analysis. Locations excluded were Algiers, L'Île-Rousse, Molivos, Azla, Dos Torres, Cala Blanca, Cap d'Artrutx, Cala de sant Vicenç, Chipiona, Dénia, El Hierro, Peniscola, Porto Torres, Santa Marinella, Formiggi beach, Monopoli, Tropea, Bizerte, Çirali Limani and Fethiye.

Preliminary BLAST searches were performed via GenBank's online nucleotide database, and given the evidence of high levels of genetic differentiation between three lineages within *A. lunulatus*, Kimura 2-parameter (K2P) and raw (p) distances were computed for the mitochondrial gene using MEGA version 6 (Tamura *et al.* 2013). The COI data set included three haplotypes for each of the three *A. lunulatus* lineages, plus 10 sequences from GenBank identified as *A. lunulatus* (KF452903.1; JQ305885.1), *Acanthonyx scutiformis* (Dana, 1851) (KC695767.1), *Acanthonyx dissimulatus* Coelho and Torres, 1993 (KC695765.1) and *Acanthonyx petiverii* Milne Edwards, 1834 (KC695771.1;

KC695773.1; KC695775.1; KC695772.1; EU682854.1; EU682855.1). Individuals from the latter three species were collected in the SW Atlantic. The 28S data set was complemented with the *A. petiverii* sequence EU682903.

Phylogenetic analysis and estimation of divergence times

Phylogenetic relationships between mtDNA haplotypes were investigated by building an haplotype network using the statistical parsimony procedure of Templeton *et al.* (1992) implemented in TCS version 3.5.1.2 (Clement *et al.* 2000). The network was plotted with tcsBU (Santos *et al.* 2016). Furthermore, two model-based methods of phylogenetic inference were applied to our data: Bayesian inference (BI) using MRBAYES 3.2.2 (Ronquist *et al.* 2012) and maximum likelihood (ML) using GARLI 2.0.1 (Zwickl 2006). The appropriate models of nucleotide substitution for the COI, 28S and concatenated data sets (COI + 28S) were obtained through JMODELTEST 2.1.7 (Darriba *et al.* 2012) using the Akaike information criterion (AIC). For COI, analyses were made using two data partitions (1 + 2, 3 codon positions), to minimize saturation effects of third positions on phylogenetic reconstructions. In the BI analysis, two separate runs were conducted for 3×10^7 generations each, and trees and parameters were sampled every 1000th generation with the heating parameter set to 0.25. Majority-rule consensus trees were estimated combining results from duplicated analyses, after discarding the first 7500 samples as burn-in. ML bootstrap analyses were performed using 1000 replicates to estimate support settings. Convergence between tree topologies was confirmed by examining log likelihood values across searches. The program SumTrees from package DendroPy (Sukumaran & Holder 2010) was used to describe the parameters of the best Garli-generated tree and to construct a majority-rule consensus tree from bootstrapped Garli replicates.

Finally, the software BEAST version 2.2.1 (Bouckaert *et al.* 2014) was used to estimate a species tree using the concatenated data set and estimate approximate divergence time between main lineages. Two independent runs were performed using MCMC simulations for 3×10^7 generations, with parameters sampled every 1000th generation. We used two substitution rates for COI (0.66% and 2.33%) estimated by Schubart *et al.* (1998) for Jamaican crabs to determine divergence times between the major *Acanthonyx* clades. We did not use the 28S data set for dating purposes as there are no estimates of substitution rates for the 28S gene of Decapods. To check the validity of a strict molecular clock for the entire data set, the likelihood ratio method implemented in the MEGA version 6 (Tamura *et al.* 2013) was used with the GTR+G model. The null hypothesis was rejected ($P < 0.05$), and therefore, the uncorrelated lognormal relaxed molecular clock prior was used. Results obtained with BEAST were checked in TRACER v1.5 (Rambaut & Drummond 2013) to determine adequate burn-in and MCMC chain mixing through ESS values. Burn-in was set to 3×10^6, corresponding to 10% of the total samples in each run. Finally, consensus trees were visualized in FIGTREE version 1.4.2 (Rambaut 2012).

Results

Morphological identification

Based on the observation of selected morphological characteristics, it was possible to distinguish at least two morphologically distinct lineages within *A. lunulatus sensu* Ng *et al.* (2008), hereafter referred to as *A. lunulatus sensu lato*. One of them included specimens that fit in the original description of Risso (1816), hereafter called *A. lunulatus sensu stricto* (or simply *A. lunulatus*), ranging from the Mediterranean to the Atlantic coasts of Northern Africa, including the Canaries and the Madeira archipelagos, but being absent from the Azores and Cape Verde. The other included specimens from the Azores and Cape Verde, which correspond to the description of *A. brevifrons* Milne-Edwards 1869, *sensu* Manning & Holthuis (1981), hereafter referred to as *A. brevifrons*. This can be distinguished from *A. lunulatus* mainly by the number of lateral lobes, bearing two instead of three in each side of the carapace (Fig. S1). It also has shorter rostral teeth, a smoother carapace and a V-shaped rostral sinus, instead of a U-shaped as in *A. lunulatus* (Manning & Holthuis 1981). Other morphological characteristics (e.g. chelipeds lobes, shape of the orbital margin or fifth periopod) were highly variable and did not allow a clear differentiation between these two forms.

Sequence data

No premature stop codons were found in the COI alignment, and no gaps were postulated. The alignment of the COI and the 28S data sets had a total length of 614 and 503 bp and included 100 and 10 unique haplotypes, respectively. The alignment of the concatenated data set had a total of 1117 bp and included 28 original, plus four outgroup species. For the 28S, some indels were found: one insertion was exclusive of a yet unidentified *A. lunulatus* lineage, and other was shared between this lineage and the outgroup *A. petiverii*. Another two deletions and an insertion were common to all *A. lunulatus sensu lato* lineages by contrast with *A. petiverii*. The program 2matrix (Salinas & Little 2014) was used to code the five indels into a binary partition with five characters. For this reason, the 28S and the concatenated data sets were extended with the indel partition.

Haplotype network reconstruction

Phylogenetic relationships between haplotypes are depicted in Fig. 2. The haplotype network reconstruction for all sequenced COI data retrieved three separate networks that could not be connected using the 95% parsimony connection limit. One network corresponds to the morphotype of *A. brevifrons* (Fig. 2A) and presents 16 relatively distant haplotypes that are all exclusive to Cape Verde and the Azores. There is a clear genetic separation of these archipelagos, as there are no shared haplotypes between them. However, the Azorean haplotypes differ from those of Cape Verde by only a few mutations. A second network (Fig. 2B), which does not correspond to any known described taxon (specimens are indistinguishable from the morphotype of *A. lunulatus*), displays 18 unique haplotypes. It has a simple star-like shape, with one very common haplotype surrounded by several low-frequency haplotypes with a maximum distance of three mutation steps. This group, hereafter called *Acanthonyx* Clade II (see Results from phylogenetic inference), is composed mostly by specimens which occur in the Atlantic coasts of northern Africa and southern Spain, with few haplotypes belonging to Mediterranean locations (Zaouia). The third network (Fig. 2C), corresponding to *A. lunulatus sensu stricto*, is highly branched with intricate patterns and presents 66 unique haplotypes. It has a big common haplogroup that contains haplotypes from most of the sampled locations, and a second less frequent haplogroup, nine mutations apart, with a similar geographical extent. The remaining haplogroups consist mostly of low-frequency and private haplotypes, separated by a single mutation step from the former two. A few haplogroups form distant branches and include only individuals from the Mediterranean with the exception of a haplotype from the islands of Selvagens. Interestingly, with only the previous exceptions, all haplotypes from Selvagens/Canaries form a slightly distinct haplogroup separated by at least six mutations from the main haplogroup, and being closely related with haplotypes from the Moroccan coasts of the Alboran Sea.

Diversity indices, tests of selective neutrality

Estimates of genetic diversity measures and neutrality tests are provided in Table S2 for *A. lunulatus*, *Acanthonyx* Clade II and *A. brevifrons*. The highest values of nucleotide

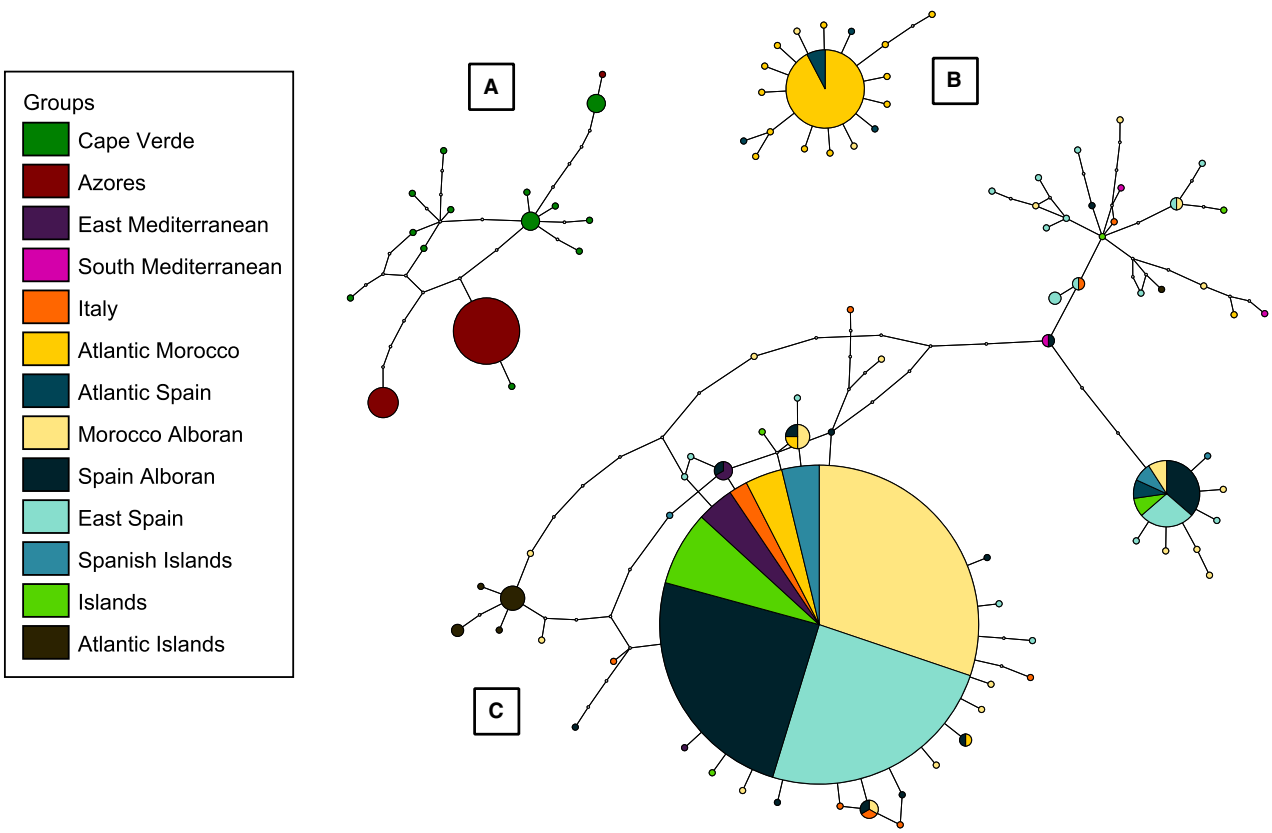

Fig. 2 Haplotype networks (95% parsimony connection limit) for all sequenced COI data. Haplotype networks correspond to (A) *Acanthonyx brevifrons*, (B) *Acanthonyx* Clade II, (C) *Acanthonyx lunulatus*. COI, cytochrome oxidase subunit I.

diversity are found in *A. lunulatus* and *Acanthonyx* Clade II. In the case of *A. brevifrons*, which is restricted to the Azores and Cape Verde, there is a marked difference in haplotype diversity between both archipelagos, despite the similar sample sizes. Whereas at Cape Verde haplotype diversity is quite high (Hd = 0.96), in the Azorean islands only three different haplotypes were found (Hd = 0.522). All species showed generally high haplotype diversity with the exception of *A. lunulatus* at the location of Al Hoceima (0.2857). Neutrality tests for *A. lunulatus* and *A. brevifrons* were mostly non-significant with the exception of two Tajima's *D* test values for Benalmadena and Selvagens and one Fu's FS test value for Mindelo, respectively. On the other hand, Fu's FS tests for the *Acanthonyx* Clade II yielded significant values for all locations with the exception of Temara, suggesting a recent expansion or a selective sweep in this clade.

Phylogenetic relationships within NE Atlantic Acanthonyx

The best common models of nucleotide substitution selected by JMODELTEST and implemented on MRBAYES, GARLI and BEAST were the GTR+G (7th) and HKY (2nd) for the COI and 28S data sets, respectively, and GTR+I (4th) and HKY (2nd) for the COI and 28S concatenated data sets (applied per partition). Phylogenetic analyses rendered trees with similar overall topologies for all data sets, differing only in the position of a few haplotypes within inner groups and also in the statistical support of the clades (Figs S2 and S3 and 3). Bootstrap support (BP) from ML and Bayesian posterior probability (PP) values were congruent with each other. The latter were usually higher than the former but it is known that PP values are typically higher than BP values (Suzuki *et al.* 2002).

A. lunulatus sensu lato was found to be monophyletic in all analyses (Fig. S3). Also, all phylogenetic analyses recovered three major clades, all of which had moderate to relatively high bootstrap support, matching the previous results from the haplotype network reconstruction: *A. brevifrons* (Clade I), *Acanthonyx* Clade II and *A. lunulatus sensu stricto* (Clade III). The *A. brevifrons* clade comprised just Cape Verde and Azorean specimens. This clade appeared as the most basal clade for mitochondrial (COI) and concatenated (COI + 28S) data sets (Figures S2 and 3), but it was weakly supported in the former. The *Acanthonyx* Clade II included Atlantic forms from Morocco and Spain and a few individuals from the Mediterranean Morocco (near the Strait of Gibraltar). This Clade was highly supported in all analysis and for all data sets, except in the ML analysis based on the mitochondrial gene (52% bootstrap value; Fig. S2). Finally, the *A. lunulatus sensu stricto* clade included haplotypes found throughout the entire study area except the Azores and Cape Verde and emerged as monophyletic in all analyses except for the 28S data set. For the COI and concatenated data sets (Figures S2 and 3), *Acanthonyx* Clade II and *A. lunulatus* appeared as sister species with moderate or high support, respectively.

The K2P- and uncorrected p-distances among individuals ranged from 0.0 to 0.18 and 0.0 to 0.16, respectively (Table S3). Overall, they were similar for the same pairwise comparison, with K2P distances being usually larger by 1%. Distances between the three identified lineages of were 0.03 between *A. lunulatus* and *Acanthonyx* Clade II and 0.07 between *A. brevifrons* and the other two. One sequence attributed by Windsor & Felder (2014) to *A. lunulatus* (KF452903) sampled at Cape Verde suggests a misidentification, as it is similar to *A. brevifrons* group (K2P = 0.01). Distances between the three clades and the outgroups (identified as *A. dissimulatus*, *A. petiverii* and *A. scutiformis*) ranged from 0.13 to 0.18. Notably, genetic distances showed only two distinct groups (American *Acanthonyx* I and II) within the outgroups, separated by a distance of 0.07. Given that the outgroups represent three named species, the present results indicate that at least one of them is wrongly identified.

Estimates of divergence times

Estimates for the time to the most recent common ancestor (TMRCA) of each clade, as well as their 95% credibility intervals, were obtained for the two different substitution rates (0.66% and 2.33%) separately. Using the lowest and highest values from the two confidence intervals obtained for each divergence event, the split between the Atlantic–Mediterranean clades of *Acanthonyx* and *A. brevifrons* is estimated to be within 0.6031 and 4.1314 Mya (node 2 in Fig. 3). The TMRCA between *Acanthonyx* Clade II and *A. lunulatus* is estimated between 0.3653 and 1.7195 Mya (node 1 in Fig. 3).

Discussion

The present work shows that the prevailing taxonomic concept of *A. lunulatus sensu lato* (following Ng *et al.* 2008) should be revised. This concept includes a single species ranging from the Mediterranean to southern Africa, including all the Macaronesian archipelagos. However, the combination of current genetic data with morphological analyses suggests this taxonomic concept includes *A. lunulatus sensu stricto*, *A. brevifrons* and a putative cryptic species or subspecies. Haplotype network of the mtDNA data produced three unlinked networks at a connection limit of 95% which, according to Hart & Sunday (2007), is a good indicator for the presence of cryptic species. With the exception of the 28S data set, phylogenetic analysis also recovered three monophyletic clades, although with varying statistical support. Average K2P- and uncorrected p-

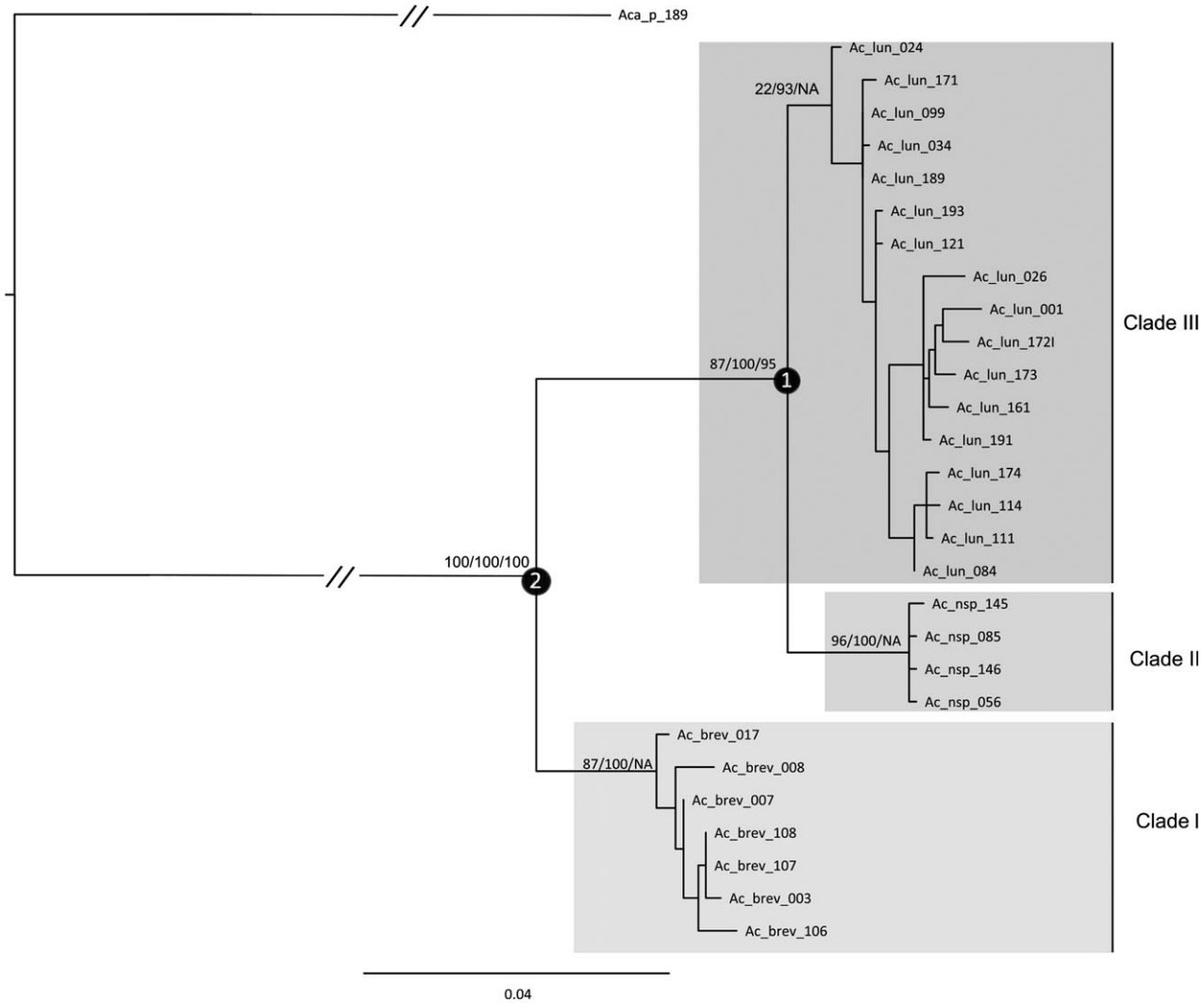

Fig. 3 Maximum-likelihood tree obtained for the concatenated data set (COI + 28S). Values at the nodes correspond to ML bootstrap support and Bayesian posterior probability for BI and the species tree, respectively. COI, cytochrome oxidase subunit I.

distances between the three linages of *Acanthonyx* provide further evidence for the current interpretation of their taxonomic status. According to Matzen da Silva *et al.* (2011), levels of intragenus divergence of the COI gene in Decapoda range from 5% to 32%. Our results show that the divergence between *A. lunulatus sensu stricto* and *A. brevifrons* is 7%, which is still within the mentioned interval, whereas between *A. lunulatus* and *Acanthonyx* Clade II it is only 3%. However, this marginal differentiation can be explained by the likely recent diversification of the group. Even though individuals belonging to each lineage were fairly similar genetically, there are sets of specimens from each lineage that clearly display higher interlineage similarities than others. This signal was observed either between *A. lunulatus* and *A. brevifrons* for the COI data set, and between the former and *Acanthonyx* Clade II for the 28 data set. Interestingly, these sets reveal no congruent geographical pattern, which may result from the high dispersal ability of *Acanthonyx*.

Our data showed that the taxonomic view that best fits the current genetic evidence is the one proposed by Manning & Holthuis (1981). Hence, all specimens from Mediterranean Sea and most of those found in the NE Atlantic localities, including the Macaronesian archipelagos of Canaries and Madeira, should be classified as *A. lunutatus*. The specimens from Cape Verde and the Azores correspond to the description of *A. brevifrons* and should be recognized as a good species. The third lineage (*Acanthonyx* Clade II) does not fit any described species or subspecies known so far and should be viewed as an unrecognized taxonomic entity or a putative cryptic species of *Acanthonyx*.

Distribution of Acanthonyx brevifrons* and *A. lunulatus

The taxonomic status of *A. brevifrons* Milne-Edwards, 1869 has never been consensual. Some authors considered it as a variety of *A. lunulatus* (e.g. Chapman & Santler 1955; Emparanza *et al.* 2007; Ng *et al.* 2008), while others viewed it as distinct species (Manning & Holthuis 1981). A possible explanation for these contrasting views stems from the superficial morphological similarities between these species, coupled with the usage of highly variable diagnostic characteristics to distinguish them. Due to the early synonymization of both species, occurrence records from the literature make it difficult to establish their actual distribution.

Milne-Edwards (1869) described *A. brevifrons* from the bay of São Vicente (Cape Verde). Later, Milne Edwards & Bouvier (1900) reported this species from the Azores (Santa Maria Island). However, in this work they also reported the presence of *A. lunulatus* in Cape Verde, citing a record from the 'Challenger' expedition without any further reference. This record can only be attributed to Miers (1886) who, based on a single immature female, noted that 'It approaches the species or variety *Acanthonyx brevifrons*, A. Milne Edwards, in the form of the front, but there are indications of three antero-lateral teeth, and the carapace, as in *A. lunulatus*, bears several tufts of setae'. Hence, Miers did not explicitly synonymize both species, an opinion later followed by many authors (e.g. Balss 1922; Monod 1933), including Bouvier (1940), in his seminal work 'Faune de France'.

D'Acoz (2001) examined Azorean specimens of what he called *A. lunulatus* and discussed the possibility of the presence of *A. brevifrons* in the archipelago because the specimens had only two well-defined lateral lobes with a small prominence hinting at the presence of a third one. Although he admitted that the specimens examined were fairly variable morphologically but formed a cohesive group, he opted to classify the Azorean specimens as *A. lunulatus* given the lack of acceptance of *A. brevifrons*. In an early work also carried out in the Azorean archipelago, Paula *et al.* (1992) identified both *A. brevifrons* and *A. lunulatus*, but remarked that the latter specimens had intermediate features between the 'true' Mediterranean *A. lunulatus* and *A. brevifrons*. All *Acanthonyx* from the Azores and Cape Verde analysed in the present work belong to a distinct genetic clade but include both the typical morphotype of *A. brevifrons* (two lateral lobes) as well as the 'intermediate' morphotype with two lateral lobes and a hint of a third median one.

Manning & Holthuis (1981) also discussed the presence of *A. lunulatus* in Cape Verde, although they did not find any direct evidence of this species in the material they collected. They did not dismiss such occurrence because their specimens were collected at 75–180 m depths, whereas previous records attributed to *A. lunulatus* were all from specimens collected at shallow waters and such non-overlapping habitats could explain their occurrence in sympatry. Specimens collected for this work at Cape Verde and the Azores all come from shallow waters (0–2 m deep) and none is genetically similar to *A. lunulatus* lineage. Hence, as there is no factual evidence of any *Acanthonyx* found in the Azores and Cape Verde with a true median lobe bearing a tuft of setae, the presence of *A. lunulatus* in both archipelagos is dismissed for the time being.

Regarding the *A. brevifrons* mtDNA haplotype network, the Azorean haplotypes do not form a distinct branch out the Cape Verde network but are related to highly differentiated haplotypes from the latter archipelago. Hence, data strongly suggest that haplotype diversity in the Azores is not created in loco, hinting to a recent colonization of this archipelago from Cape Verde. Whether this colonization is historical or contemporary (eventually human-mediated) cannot be answered by the current data given the high haplotype and nucleotide diversity of COI in *A. brevifrons*, and lack of spatial coverage.

***A cryptic lineage of Acanthonyx* in south-west Iberia and NE Africa**

Several individuals from the Atlantic coasts of south-east Spain and north-east Morocco, and some Moroccan Mediterranean localities, formed a distinct, monophyletic and well-supported clade. Their COI haplotype network displayed a characteristic star-like structure, suggesting a recent demographic expansion. This conclusion is supported by the Fu's Fs neutrality tests, which were significant for all sampling sites but Temara (Morocco). A detailed morphological analysis of all individuals of this clade using all morphological traits described by Manning & Holthuis (1981) and Zariquiey Alvarez (1968) did not reveal any characteristic that allowed their distinction from *A. lunulatus*. Comparison with the description of a southern Atlantic species (Chace, 1966) known from the islands of Ascension and Saint Helena – *Acanthonyx sanctaehelenae* Chace, 1956 – was also inconclusive.

Both K2P- and uncorrected p-distances between *A. lunulatus* and *Acanthonyx* Clade II are within intraspecies diversity in Majoidea (Matzen da Silva *et al.* 2011), but a 3% divergence level has already been accepted as a threshold for cryptic species in other crustaceans (Radulovici *et al.* 2009). Notwithstanding, the strongest genetic difference between *Acanthonyx* Clade II and the other two lineages was detected in the nuclear gene through the presence of indels. *Acanthonyx* Clade II shares a deletion with *A. lunulatus* and *A. brevifrons* when compared to the outgroup *A. petiverii*, but differs from the former two by having two insertions, one if which is also shared with

A. petiverii. Furthermore, all three lineages of *A. lunulatus sensu lato* share an insertion which lacks in *A. petiverii.* Usage of indels in phylogenetic analysis should be taken with caution but it is important not to ignore this kind of information because it can improve the interpretation of the results (Zhang & Hewitt 2003). Nuclear ribosomal genes are known to be highly uniform within species, but may vary significantly between species (Eickbush & Eickbush 2007). We found no evidence of heterozygosity in the 28S of both *A. lunulatus* and *Acanthonyx* Clade II. The absence of shared haplotypes at cytoplasmic and nuclear genes is good evidence of reproductive isolation, which is surprising given that the two lineages occur in sympatry, and cryptic species tend not to co-occur (Vodă *et al.* 2015). However, a greater sampling effort at the entrance of the Mediterranean and the usage of more molecular markers will help to determine the taxonomic status of this distinct *Acanthonyx* group.

The taxonomy of the genus Acanthonyx

The genus *Acanthonyx* is known to occur worldwide, but the present work, although focused only on the NE Atlantic region using a presumably single species, unveils potential problems with the taxonomy of this morphologically variable group of spider crabs. On the course of the phylogenetic analysis, we noticed that a COI sequence published in GenBank (KF452903), which was attributed to *A. lunulatus*, differed by 7% from our own sequences. This sequence was used by Windsor & Felder (2014) as an outgroup for the phylogenetic analysis of the family Mithracidae (Epialtidae) and corresponds to a specimen sampled from Cape Verde. In the light of the current molecular data, this specimen belongs to *A. brevifrons* (K2P distance of 1%). Whether the authors explicitly adopted the taxonomic concept of Ng *et al.* (2008) or were unaware of the work of Manning & Holthuis (1981) remains to be known.

The use of K2P- and uncorrected p-distances of COI to estimate average levels of divergence between good species of *Acanthonyx* also revealed some problematic issues with sequences deposited on GenBank. For example, while studying American epialtids, Gomes (2013) found two main lineages within *Acanthonyx* species separated by approximately 7% of genetic divergence. One included most of the individuals identified as *A. petiverii* from Central and South America, together with a specimen identified as *A. dissimulatus* and another identified as *A. scutiformis* from Brazil. The other lineage included one specimen identified as *A. petiverii* collected at a Mexican locality in the Gulf of Mexico (KC695766), one specimen identified as *A. dissimulatus* collected in the Caribbean (KC695765) and a specimen identified as *A. petiverii* from the Gulf of Mexico (EU682854), which was used by Hultgren & Stachowicz (2008) on the phylogeny of the Majoidea. She suggested the synonymization of *A. dissimulatus* and *A. scutiformis* with *A. petiverii*, although the observed levels of genetic divergence clearly point to the presence of two species and thus to possible misidentifications. Interestingly, in their redescription of *A. petiverii*, Emparanza *et al.* (2007) compared the specimens collected in Chile with the descriptions of Chace (1966) for the Martinique and the Virgin Islands. They noted consistent morphological differences between both groups, namely on the disposition of tubercles on the carapace, but they refrained from discussing the conspecific status of Pacific and eastern Atlantic *A. petiverii* populations without a more comprehensive morphological examination or new evidence.

Most of the taxonomic problems mentioned result from the difficulty in establishing good diagnosis for the morphologically highly variable genus *Acanthonyx*, and a comprehensive revision of this genus is still needed. Although many details have been unravelled for the *Acanthonyx* from north-east Atlantic and Mediterranean, some doubts still remain regarding the distribution of *A. lunulatus sensu stricto*. Given the levels of genetic differentiation observed, its distributional range – from the Mediterranean southwards to Namibia – should be investigated. There are few known records of *A. lunulatus* for the Gulf of Guinea, but most were recently placed into two different species: *A. depressifrons* Manning & Holthuis, 1981 and *A. minor* Manning & Holthuis, 1981. Hence, the presence of *A. lunulatus* in south-east Africa is dubious, although molecular evidence for other Majoidea species with similar larval duration shows that such a wide distribution is possible (Sotelo *et al.* 2009).

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Supporting Information

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

Fig. S1. Dorsal view of *Acanthonyx lunulatus* (A) and *Acanthonyx brevifrons* (B), showing the two main morphological differences between them. The specimens of *A. lunulatus* have three lateral lobes on each side of the carapace and exhibit a U-shaped rostral sinus (A); *A. brevifrons* shows only two lateral lobes and has a V-shaped rostral sinus (B).

Fig. S2. Maximum-likelihood tree obtained for the mitochondrial COI gene. Values at the nodes correspond to ML bootstrap support and Bayesian posterior probability, respectively.

Fig. S3. Maximum-likelihood tree obtained for the 28S rRNA gene. Values at the nodes correspond to ML bootstrap support and Bayesian posterior probability, respectively.

Table S1. *Acanthonyx* species, SeqDB identification for the voucher, code of individual, locality, coordinates and NCBI sequence accession numbers for each specimen included in the analysis.

Table S2. Estimates of genetic diversity and neutrality tests for the mtDNA COI gene for each sampling site.

Table S3. COI K2p-distances (below the diagonal) and uncorrected p-distances (above the diagonal) between *Acanthonyx brevifrons*, *Acanthonyx* clade II, *Acanthonyx lunulatus* and outgroups (*A. dissimulatus*; *A. petiverii* and *A. scutiformis*). Distances above 0.05 are depicted in bold.