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ORIGINAL PAPER

Re-evaluation of the *Doriopsilla areolata* Bergh, 1880 (Mollusca: Opisthobranchia) subspecies complex in the eastern Atlantic Ocean and its relationship to South African *Doriopsilla miniata* (Alder & Hancock, 1864) based on molecular data

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Abstract *Doriopsilla areolata* is a nudibranch sea slug with a broad geographic range, spanning from mainland Spain (southwestern Europe) to Angola (southwestern Africa) in the eastern Atlantic, and is also found in the Caribbean. This species was divided into three subspecies: *D. areolata areolata* (Europe to Cape Verde), *D. areolata albolineata* (Ghana to Angola), and *D. areolata nigrolineata* (Caribbean), each with a discrete range. This paper uses phylogenetic and population genetics tools to determine whether the subspecies scheme is supported by molecular data. Additionally, specimens of *Doriopsilla miniata* from South Africa, a species that is hypothetically closely related to *D. areolata*, is also included in the analysis, primarily because of the morphological similarities between *D. areolata* and *D. miniata*. Results from AMOVA, Bayesian, and pairwise analyses revealed that the molecular data do not support the proposed subspecies scheme and suggest that South African populations identified as *D. miniata* belong to *D. areolata*.

Keywords Population genetics · Phylogenetics · 16S · H3 · Nudibranchia · Biogeography

Introduction

The nudibranch mollusk *Doriopsilla areolata* was described by Bergh (1880) from the Adriatic Sea. This species has an unusually broad geographic range, covering most of the eastern Atlantic from northern Spain to Angola, as well as the Caribbean Sea (Valdés and Ortea 1997; Valdés and Hamann 2008). Valdés and Ortea (1997) separated this species into three distinct subspecies (each with a discrete range) based on some morphological and coloration differences. According to this scheme, *Doriopsilla areolata areolata* Bergh, 1880 ranges from the North coast of Spain and the Mediterranean Sea to the Cape Verde islands, *D. areolata albolineata* Edmunds, 1968 is found in Ghana, São Tomé and Angola on the west coast of Africa (Edmunds 1968; Valdés and Ortea 1997), and *D. areolata nigrolineata* Meyer, 1977 is known only from the Caribbean coast of Panama (Meyer 1977; Valdés & Ortea 1997). Although *Doriopsilla nigrolineata* was originally described by Meyer (1977) as a distinct species, Valdés and Ortea (1997) considered it to be a subspecies of *D. areolata*. More recently, Valdés and Hamann (2008) resurrected *D. nigrolineata* as a distinct species from *D. areolata* because of numerous anatomical differences. However, Valdés and Hamann (2008) also reported the presence of *D. areolata* in the Caribbean. Moreover, Calado et al. (2012) demonstrated that *D. areolata* is a planktotrophic developer that places an extensive amount of resources to produce a large number of small eggs. Therefore, this species has the potential to disperse long distances, which calls into question the existence of genetically isolated populations or subspecies in areas with no evident barriers to dispersal.

Another similar, possibly closely related, species is *Doriopsilla miniata*, described by Alder and Hancock (1864) from India. This species has also been reported from South Africa (Gosliner 1987) and temperate Australia (Burn 2006), giving it an unusual geographic range that includes

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both temperate and tropical locations with large gaps in between populations. Descriptions of the egg masses of specimens of *D. miniata* from southern Australia (Rose 1985) revealed that they are direct developers, which makes them very unlikely long-distance dispersers. Unless human introductions are responsible for this broad range, it is difficult to accept that populations from South Africa, India, and Australia belong to the same species. Valdés and Ortea (1997) suggested that *D. miniata* from South Africa could constitute another subspecies of *D. areolata*, but did not provide any morphological evidence.

The hypothetical existence of distinct populations (or subspecies) in *Doriopsilla areolata* has never been tested using molecular data. Because of the unusual range of this species, unraveling the genetic structure of *D. areolata* has broader implications to understand biogeographic patterns and barriers to dispersal in the Atlantic Ocean. In this study, we will focus on the two eastern Atlantic subspecies, *D. areolata areolata* and *D. areolata albolineata*, and populations of *D. miniata* from South Africa. Specimens of *D. areolata nigrolineata* were not included in the analyses as this subspecies is extremely rare in the field and all available specimens were preserved in formalin, making them unsuitable for molecular work. Using molecular information from the mitochondrial (16S) and nuclear (H3) gene data obtained from specimens covering most of the eastern Atlantic, we will attempt to determine whether the subspecies scheme is substantiated. Numerous attempts to amplify COI from museum specimens using different primers were unsuccessful.

Materials and methods

Source of specimens

DNA from 18 specimens of *Doriopsilla areolata* covering the entire range of the species in the eastern Atlantic was extracted but in only eight of them was it successfully amplified and sequenced (Table 1). Specimens were obtained from the collections of the Museo Nacional de Ciencias Naturales in Madrid, Spain (MNCN) and the Natural History Museum of Los Angeles County, California, USA (LACM). All specimens were preserved in 70–75 % EtOH. In addition, three specimens of *Doriopsilla miniata* from South Africa, obtained from the

Table 1 List of *Doriopsilla* specimens used in the study; including locality, voucher number, and GenBank Accession numbers

Species	Locality	Voucher no.	GenBank accession no.	
			16S	H3
D. janaina	Perú	CPIC 00590	KC171022	KC171034
D. areolata areolata	Cape Verde	MNCN 15.05/23781	KC171027	–
D. areolata areolata	Cape Verde	MNCN 15.05/23784	–	–
D. areolata areolata	Málaga, Spain	MNCN 15.05/23783	–	–
D. areolata areolata	Cantabria, Spain	MNCN 15.05/18276	–	–
D. areolata areolata	Girona, Spain	MNCN 15.05/23766	KC171023	KC171040
D. areolata areolata	Las Palmas, Spain	MNCN 15.05/23782	KC171026	KC171037
D. areolata areolata	Granada, Spain	MNCN 15.05/17709	–	–
D. areolata areolata	Cádiz, Spain	LACM 2002-88.24	–	–
D. areolata areolata	Cádiz, Spain	LACM 2001-10.3	KC171024	KC171035
D. areolata areolata	Cádiz, Spain	LACM 2001-10.4	KC171025	KC171036
D. areolata albolineata	São Tomé Island	MNCN 15.05/23787	–	–
D. areolata albolineata	Ghana	MNCN 15.05/23774	–	–
D. areolata albolineata	Ghana	MNCN 15.05/23773	–	–
D. areolata albolineata	Angola	MNCN 15.05/23792	–	–
D. areolata albolineata	Angola	MNCN 15.05/23786	–	–
D. areolata albolineata	Angola	MNCN 15.05/23771	KC171032	–
D. areolata albolineata	Angola	MNCN 15.05/23784	KC171033	KC171039
D. areolata albolineata	Angola	MNCN 15.05/23789	KC171031	KC171038
D. miniata	W. Cape, South Africa, Atlantic	CASIZ 176418	KC171028	KC171041
D. miniata	W. Cape, South Africa, Atlantic	CASIZ 176933	KC171029	KC171042
D. miniata	W. Cape, South Africa, Atlantic	CASIZ 176370	KC171030	KC171043

Department of Invertebrate Zoology and Geology of the California Academy of Sciences in San Francisco, California, USA (CASIZ), were sequenced, as well as one specimen of *Doriopsilla janaina* Marcus & Marcus, 1967 from Perú, obtained from the California State Polytechnic University Invertebrate Collection (CPIC). One sequence of *Doriopsilla albopunctata* (Cooper, 1863) was obtained from GenBank (AF430354). *Doriopsilla janaina* and *D. albopunctata* were used for outgroup comparison.

DNA extraction

DNA extraction was performed using either a hot Chelex® protocol or DNeasy Blood and Tissue Kits (Qiagen). Approximately 1–3 mg tissue was taken from the animals and cut into fine pieces for extraction for each of the three protocols. For the Chelex® extraction, the tissue was rinsed and rehydrated using 1.0 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 20 min. A 10 % (w/v) Chelex® 100 (100–200 mesh, sodium form; Bio-Rad) was prepared using TE buffer. After rehydration, the mixture was then centrifuged, 975 mL of the supernatant was removed, and 175 mL of the Chelex® solution was added. Samples were then heated in a 56 °C water bath for 20 min, then heated in a 100 °C heating block for 8 min, and the supernatant was used for PCR. The DNeasy protocol supplied by the manufacturer was followed, with some modifications. The elution step was modified such that the first elution was collected using 100 μL of Buffer AE and was allowed to incubate at room temperature for 5 min. In a new test tube, a second elution step was conducted using 200 μL of Buffer AE and was also allowed to incubate at room temperature for 5 min. The first elution, or in some cases the second elution, was used for PCR. The reason why multiple extraction protocols were implemented was due to the fact that some tissue samples could not be amplified when using a particular extraction method. The use of the DNeasy extraction protocol was only used as an alternative to the Chelex® extraction when it became apparent that the DNA could not be successfully extracted using this latter technique.

Primers

Colgan's universal H3 primers (Colgan et al. 1998) were used to amplify the regions of interest for all specimens and Palumbi's universal 16S primers (Palumbi 1996) were used to amplify the regions of interest for some specimens (Table 2). However, internal primers for 16S designed for another group of opisthobranchs (Ornelas-Gatdula et al. 2011) were used resulting in additional partial sequences for some specimens.

PCR amplification and sequencing

The master mix was prepared using 34.75 mL H_2O, 5 mL Buffer B (ExACTGene; Fisher Scientific), 5 mL 25 mM $MgCl_2$, 1 mL 40 mM dNTPs, 1 mL 10 mM primer 1, 1 mL primer 2, 0.25 mL 5 mg/mL Taq, and 2 mL extracted DNA. Reaction conditions for H3 and 16S rRNA were as follows: an initial denaturation for 2 min at 94 °C, 35 cycles of (1) denaturation for 30 s at 94 °C, (2) annealing for 30 s at 50 °C, and (3) elongation for 1 min at 72 °C, and a final elongation for 7 min at 72 °C. PCR products yielding bands of appropriate size (approximately 375 bp for H3 and 195 bp for the 16S fragments) were purified using the Montage PCR Cleanup Kit (Millipore).

Cleaned PCR samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Each primer was diluted to 2.0 pmol/mL to send out for sequencing with the PCR products. PCR products were diluted to 6.0 ng/mL for H3 and 7.5 ng/mL for 16S. These samples were sequenced at the City of Hope DNA Sequencing Laboratory (Duarte, CA) using chemistry type BigDye V1.1, which is used for fragments less than 500 bp.

Sequence analyses

Sequences for each gene were assembled and edited using Geneious Pro 4.7.4 (Drummond et al. 2009). Geneious was also used to extract the consensus sequence and to construct the alignment for each gene using the default parameters.

Table 2 Forward (*F*) and reverse (*R*) PCR primers used to amplify regions of the nuclear H3 gene and mitochondrial 16S gene

Name	Sequence 5′–3′	Source
H3		
HexAF (F)	ATG GCT CGT ACC AAG CAG ACG GC	Colgan et al. (1998)
HexAR (R)	ATA TCC TTG GGC ATG ATG GTG AC	Colgan et al. (1998)
16S rRNA		
16Sar-L (F)	CGC CTG TTT ATC AAA AAC AT	Palumbi (1996)
16Sbr-H (R)	CCG GTC TGA ACT CAG ATC ACG T	Palumbi (1996)
16Sar-FAP (F)	AAA GAC GAG AAG ACC CTT AGA GTT TT	Ornelas-Gatdula et al. (2011)
16Sbr-FAP (R)	AAA ACT CTA AGG GTC TTC TCG TCT TT	Ornelas-Gatdula et al. (2011)

The sequences were not trimmed after alignment. A total of 328 bp for H3 and between 409 and 414 bp for both 16S fragments were used for the phylogenetic analyses. A combined analysis was conducted using all taxa and both genes concatenated (Fig. 1).

The levels of saturation for each gene were investigated using the substitution saturation test developed by Xia et al. (2003) and Xia and Lemey (2009) implemented in the program DAMBE (Xia and Xie 2001).

To assess whether H3 and 16S have significantly conflicting signals, the incongruence length difference (ILD) test (Mickevich and Farris 1981; Farris et al. 1994), implemented in PAUP*4.0 as the partition homogeneity test (Swofford 2002), was conducted for both genes combined.

A Bayesian analysis was conducted using both genes concatenated. The Akaike information criterion (Akaike 1974) was executed in MrModeltest v.2.3 (Nylander 2004), using PAUP*4.0 (Swofford 2002), to determine the best-fit model of evolution for each gene (Table 3). The analysis was implemented in MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001), with partitioning by gene (unlinked). The Markov chain Monte Carlo analysis was run with two runs of six chains for 10 million generations, with sampling every 100 generations, and a default burn-in of 25 %. Clades with posterior probabilities ≥0.95 (α=0.05) were considered significant (Alfaro et al. 2003).

Population genetic analyses

The population structure for the 16S haplotypes of Eastern Atlantic *Doriopsilla* spp. was analyzed by performing a two-level hierarchical analysis of molecular variance (AMOVA) implemented in Arlequin 3.512 (Excoffier and Lischer 2010). Samples were allocated to populations according to their geographic location and the following three groups were defined according to subspecies: "Europe" (*Doriopsilla areolata areolata*), "Angola" (specimens of *D. areolata albolineata*), and "South Africa" (*D. miniata*). The genetic differentiation between populations of was analyzed using an F_{ST} pairwise analysis also implemented in Arlequin 3.5 A haplotype network for 16S was constructed using the program TCS1.21 (Clement et al. 2000).

Results

The saturation analyses showed insignificant levels of saturation for H3 and 16S (lss<lss.c, p=0.000) even when the third codon positions of H3 were analyzed independently. The ILD test showed no significant conflicting signals between H3 and 16S (P=0.65)

In the Bayesian consensus tree, there is very little structure and most groups are poorly supported (Fig. 1). The one

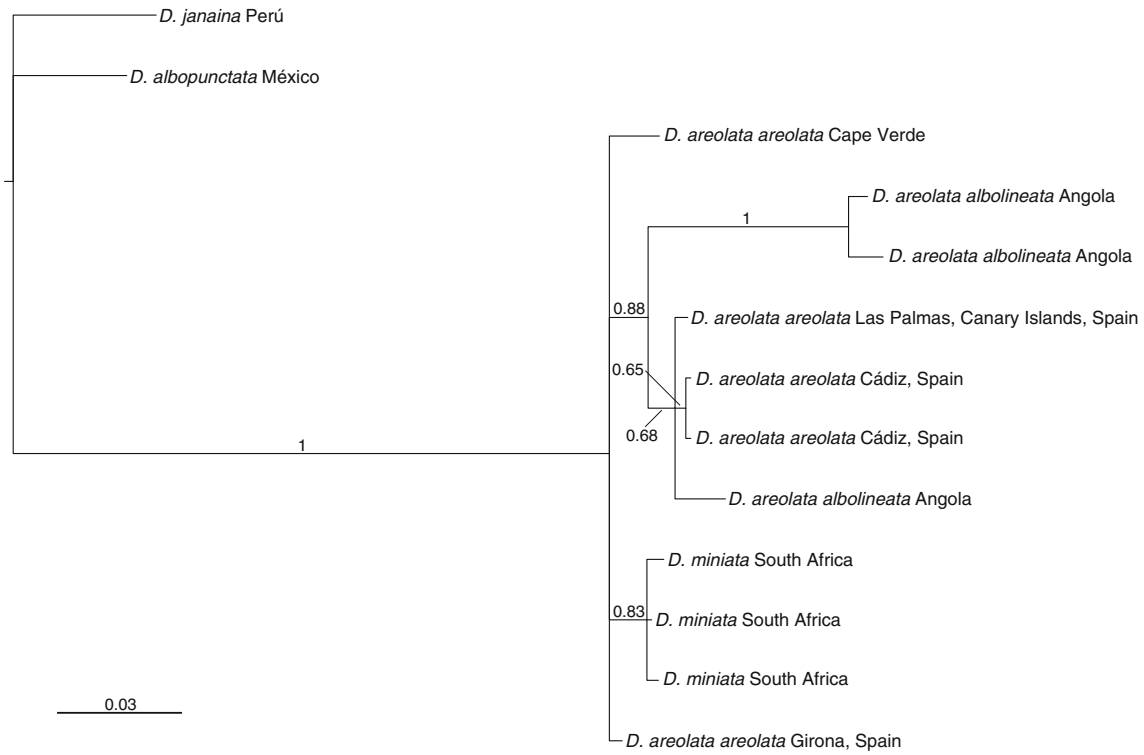

Fig. 1 Bayesian consensus tree of concatenated alignments of the 16S and H3 genes. Values are posterior probabilities (>0.95 considered significant)

Table 3 Summary of each dataset used for analysis with the best-fit evolutionary models and estimated parameters

Parameters	H3	16S
No. of specimens used in the study	9	12
No. of included characters	328	422
Best-fit model	HKY	GTR+G
Frequency A	0.2520	0.3189
Frequency C	0.3257	0.1494
Frequency G	0.2520	0.2073
Frequency T	0.1703	0.3245
R-matrix [A–C]	–	0.0000
R-matrix [A–G]	–	10.1153
R-matrix [A–T]	–	3.5217
R-matrix [C–G]	–	1.7138
R-matrix [C–T]	–	12.0756
R-matrix [G–T]	–	1.0000
Γ shape (G)	–	0.1569
Proportion of Invariant Sites (I)	0	0

exception is a group that contains only specimens of *D. areolata albolineata* from Angola (posterior probability= 1); however, the other specimen from Angola sequenced is not included in this clade. Another clade includes two specimens of *D. areolata areolata* from mainland Spain and one from the Canary Islands, Spain, as well one specimen of *D. areolata albolineata* from Angola, but is poorly supported (posterior probability=0.68). The tree suggests that *D. miniata* from South Africa forms a monophyletic group, but also poorly supported (posterior probability=0.83). In summary, the phylogenetic analysis provides no evidence of reciprocal monophyly between specimens identified as *D. areolata* or *D. miniata* (from South Africa) nor is there evidence of a subspecies or population structure among specimens identified as *D. areolata areolata* or *D. areolata albolineata*.

In the AMOVA analysis, among group differentiation explained 52.39 % of the covariance component (Φ_{CT}= 0.52388, P=0.06668, based on 16,002 permutations), among populations within groups explained −5.60 % (Φ_{SC}=0.46786, P=0.10249, based on 16,002 permutations) and within population differentiation explained 53.21 %. The pairwise F_{ST} values ranged from −0.16667 to 1.00000 and no values were significantly higher than zero.

Figure 2a illustrates the geographic distribution of H3 alleles, showing that there are only three H3 alleles present in all of the populations of both *D. areolata* and *D. miniata*. One is present in Mediterranean Spain and South Africa, the second is found in Atlantic Spain and Angola, and there is a third allele unique to Angola. This distribution of alleles is indicative of no geographic structure for this gene. For 16S, there are a total of seven haplotypes, and there is also little geographic structure. As indicated by the 16S haplotype network in Fig. 2b, the haplotypes found in South Africa are closely related to one found in Mediterranean Spain. Also similar to H3, the haplotype of one of the specimens found in Angola is more closely related to haplotypes from Atlantic Spain than to those of the other specimens from Angola.

Discussion

The phylogenetic analysis results indicate that the previous subspecies scheme for *Doriopsilla areolata* proposed by Valdés and Ortea (1997) is unsupported by all analyses. These results are further substantiated by the AMOVA and F_{ST} pairwise analyses. The AMOVA results indicate that most of the genetic variation is within populations, suggesting that the three hypothetical populations or subspecies are not significantly different. The F_{ST} pairwise analysis was consistent with the AMOVA results, as no pairwise comparisons between populations were significantly different from zero. Although the sample size was small for all populations studied, there is very little variation between populations even in the less conserved 16S, which indicates some level of gene flow between them. The results of the analysis indicate that *D. miniata* from South Africa should probably be transferred to *D. areolata*, based on both the genetic analyses and the presence of the same H3 allele found in both South Africa and Mediterranean Spain, as well as the close similarities between the 16S haplotypes found in both locations.

Even though the results of this study suggest a certain level of gene flow across all populations studied, for 16S, which is the least conserved of the two genes, there are closely related haplotypes present only in certain populations. For example, three haplotypes in Fig. 2b (red) are only present in Spain (including the Canary Islands) and three haplotypes (dark blue) are only present in South Africa. One possible explanation for this pattern is that there are barriers affecting gene flow, but not to such an extent that it prevents gene flow altogether. Another possibility is that such barriers were active in the past, but dissipated over time, allowing more gene flow across them. For example, the Congo River, just north of Angola, has been studied in relation to fluctuations in the intensity of freshwater flow into the Atlantic Ocean. Data seem to indicate that there have been fluctuations in the past of larger amounts of fresh water into the ocean causing periods of time in which gene flow may have been less likely (Uliana et al. 2002). Such a barrier could have resulted in a certain level of isolation between southern African populations and the rest of the range. A larger sample size is necessary to evaluate the detailed populations structure of *D. areolata* and fully understand

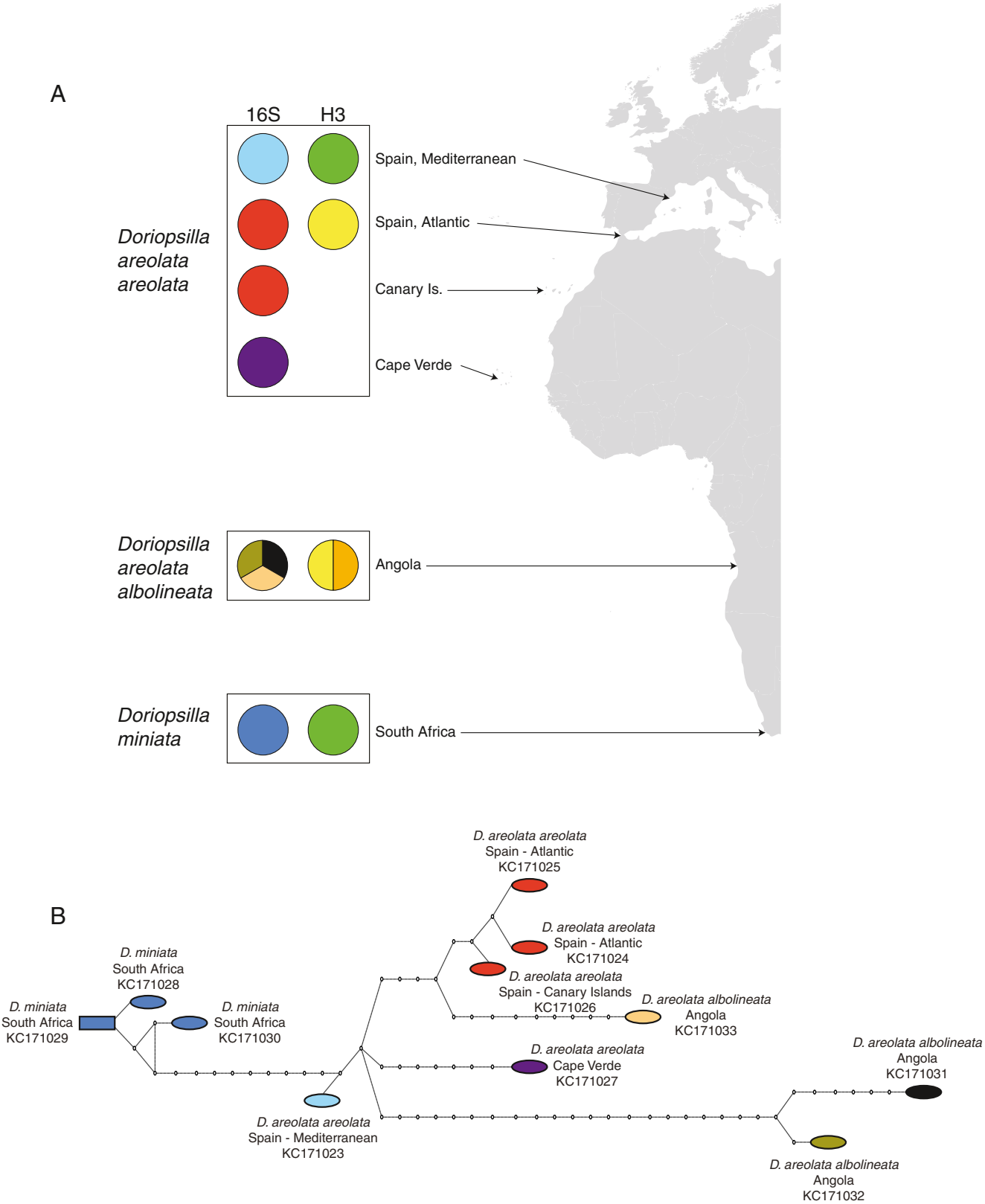

Fig. 2 **a** The geographic distribution of haplotypes and alleles for 16S and H3, respectively. For H3, colors indicate individual alleles, and for 16S, colors indicate haplotype groups identified in the haplotype network analysis (**b**). Each haplotype group includes haplotypes within three or less nucleotide substitutions. **b** Haplotype network for 16S with GenBank accession numbers for each sequence

the degree of genetic isolation between different populations of this species.

Several other studies have dealt with the population genetics of northeastern Atlantic marine organisms including samples from North Africa (Duran et al. 2004; Lessios et al. 2001; Pérez-Losada et al. 2007; Xavier et al. 2011), but none cover species with a range similar to that of *D. areolata*, which extends from Europe to southern Africa. Duran et al. (2004) found significant genetic differentiation between specimens of the sea urchin *Paracentrotus lividus* from the Atlantic and Mediterranean basins, probably due to restricted gene flow across the geographical boundary of the Strait of Gibraltar, but found no evidence of differentiation between samples from the Canary Islands and mainland Spain. On the contrary, Lessios et al. (2001) determined that populations of *Diadema antillarum* from the eastern Atlantic ranged from the Canary Islands and Madeira to São Tomé in central Africa, and there was no genetic differentiation between them. Similarly, Pérez-Losada et al. (2007) did not find population boundaries in the cuttlefish *Sepia officinalis* across most of its range (including the Atlantic coast of Europe, the Mediterranean, Mauritania, and the Canary Islands), except for the Aegean–Ionian stock. However, Xavier et al. (2011) found three distinct clades in the marine isopod *Stenosoma nadejda* distributed in the Iberian Atlantic plus the Alboran Sea, the western Mediterranean, and the Atlantic coast of Africa. The complex biogeographic history of the northeastern Atlantic with population fragmentation due to isolation of water masses during the Pleistocene (Pérez-Losada et al. 2007) has resulted in different patterns of diversification in different taxa, making generalizations on the biogeography of the region difficult. The present paper is one of the first attempts to examine the population structure and phylogenetics of a species with a much broader range and appears to suggest that in the case of *D. areolata* there is little evidence of genetic differentiation between distant populations.

The results of this study need to be substantiated with a larger sample size and specimens from additional locations in West Africa. This would allow us to determine the extent of gene flow in *D. areolata* along the eastern Atlantic and understand how possible biogeographical barriers affect dispersal in this species. Further specimens of *D. miniata* from South Africa, India, and Australia would also allow us to better understand the geographic structure of *D. miniata* and determine whether *D. areolata* extends into the Indo-Pacific or *D. miniata* from South Africa was simply a misidentified population of *D. areolata*. Additional genes, e.g., COI, might also provide further evidence to better resolve the relationships between these three populations. However, it was difficult to obtain additional specimens suitable for molecular work from the western coast of Africa, which prevent us from including a larger sample size.

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