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Molecular barcode and morphological analyses reveal the taxonomic and biogeographical status of the striped-legged hermit crab species *Clibanarius sclopetarius* (Herbst, 1796) and *Clibanarius vittatus* (Bosc, 1802) (Decapoda : Diogenidae)

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Abstract. The taxonomic status of the species *Clibanarius sclopetarius* (Herbst, 1796) and *Clibanarius vittatus* (Bosc, 1802), which have sympatric biogeographical distributions restricted to the western Atlantic Ocean, is based only on differences in the colour pattern of the walking legs of adults. Their morphological similarity led to the suggestion that they be synonymised. In order to investigate this hypothesis, we included species of *Clibanarius* Dana, 1892 in a molecular phylogenetic analysis of partial sequences of the mitochondrial 16S rDNA gene and the COI barcode region. In addition, we combined the molecular results with morphological observations obtained from several samples of these two species. The genetic divergences of the 16S rDNA and COI sequences between *C. sclopetarius* and *C. vittatus* ranged from 4.5 to 5.9% and 9.4 to 11.9%, which did not justify their synonymisation. Differences in the telson morphology, chela ornamentation, and coloration of the eyestalks and antennal peduncle provided support for the separation of the two species. Another interesting result was a considerable genetic difference found between populations of *C. vittatus* from Brazil and the Gulf of Mexico, which may indicate the existence of two homonymous species.

Additional keywords: 16S rDNA, Anomura, barcoding COI, molecular systematics, western Atlantic.

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Introduction

The genus *Clibanarius* Dana, 1892 includes species of hermit crabs that are very common in intertidal and shallow-water areas of tropical and temperate seas (Forest and Saint-Laurent 1968; Hazlett 1981; Leite *et al*. 1998; Melo 1999; Mantelatto *et al*. 2010); 59 species are presently recognised (McLaughlin *et al*. 2010). Some taxonomic doubts exist with respect to species of this genus that are closely allied to one another, and in some cases, the morphological similarity has raised questions as to their status as separate species (McLaughlin *et al*. 2010).

None of these cases has yet been clarified. One example concerns two Atlantic species, *Clibanarius sclopetarius* (Herbst, 1796) and *Clibanarius vittatus* (Bosc, 1802), and this taxonomic doubt stems from two main aspects: their sympatric biogeographical distributions (Sánchez and Campos 1978; Melo 1999; Nucci 2002) and their very similar general morphology (Holthuis 1959; Forest and Saint-Laurent 1968; Sánchez and Campos 1978; Nucci 2002). The two species are restricted to the western Atlantic Ocean, but the current known distribution of *C. vittatus* is slightly broader than that of *C. sclopetarius*. The former occurs in the eastern USA (from Virginia to Florida), the Gulf of Mexico, Antilles, Colombia, Venezuela, Suriname, Guyanas, and Brazil (from Pará to Santa Catarina) and the latter occurs in the eastern USA (Florida), Antilles, Panama, Colombia, Venezuela, Suriname, Guyanas, and Brazil (from Piauí to Santa Catarina) (Forest and Saint-Laurent 1968; Sánchez and Campos 1978; Melo 1999; Nucci 2002).

Morphological differences between the two species, based on the shape of the rostrum, the ratio between the length and width of the last segment of the antennal peduncle, the ratio between the anterior width of the cephalothoracic shield and the length of the eyestalk, and the colour pattern of the walking legs, were mentioned by Holthuis (1959). However the first three characters were refuted by Forest and Saint-Laurent (1968), who compared one adult male of each species. Therefore, only the colour pattern of the walking legs is currently used to distinguish the two taxa (*sensu* Forest and Saint-Laurent 1968), although this difference is lost when the animals are preserved in alcohol or formalin for long

periods (Forest and Saint-Laurent 1968; FLM pers. obs.), which makes it difficult to identify preserved specimens. As intraspecific phenotypic plasticity in coloration patterns is common within some crustacean groups, including closely related hermit crab species (Ball and Haig 1974; Bauer 1981; Kuris *et al*. 1987; Wilson 1987; Moraes-Riodades and Valenti 2004; Mantelatto *et al*. 2006; Reuschel and Schubart 2007; Malay and Paulay 2010), and colour-pattern differences do not necessarily indicate different species (Knowlton and Mills 1992), it seemed appropriate to propose the synonymy of *C. vittatus* and *C. sclopetarius.*

In general, past systematic studies on hermit crabs have been based on morphological characters (Mantelatto *et al*. 2009) and only recently have molecular tools been applied to solve questions of species status (Mantelatto *et al*. 2006, 2009; Matzen da Silva *et al*. 2011) or to determine lower levels of phylogenetic relationships (Morrison *et al*. 2002; Young *et al*. 2002; Zaslavskaya *et al*. 2009), such as the relationships among hermit crab species (Mantelatto *et al*. 2006; Matzen da Silva *et al*. 2011). Considering that species status is still difficult to resolve using morphological criteria alone, and there has been no previous attempt to resolve questions of evolutionary relationships among these species by means of molecular analysis, the use of this tool seemed a valid and certainly unprecedented attempt to define the taxonomic status of these two morphologically similar species, *C. vittatus* and *C. sclopetarius*.

The COI mitochondrial gene has been used as an effective molecular marker to solve taxonomic and systematic problems (Mathews *et al*. 2002; Macpherson and Machordom 2005; Mathews and Anker 2009; Ng *et al*. 2010; Puillandre *et al*. 2011), including those related to hermit crabs (Young *et al*. 2002; Matzen da Silva *et al*. 2011). Specifically, the COI barcode region can be very efficient for discriminating species, revealing genetic discontinuities between them and sometimes clarifying problems of synonymy (Hebert and Gregory 2005), and this property is applicable to decapod species (Costa *et al*. 2007; Matzen da Silva *et al*. 2011). Therefore, as part of an ongoing fine-scale biodiversity initiative, we included barcoding sequences of the cytochrome *c* oxidase I (COI) of selected species of *Clibanarius* in a molecular analysis to check the hypothesis of synonymy between *C. vittatus* and *C. sclopetarius*. Partial sequences of another mitochondrial gene, 16S rDNA, were also used. This gene has conserved regions that allow interspecific analysis, including for decapod species (Schubart *et al*. 2000*a*; Mantelatto *et al*. 2007). The COI gene is less conserved, being also useful for population-level studies (Harrison 2004). Additionally, we performed a morphological study based on the traditional taxonomic characters, as well as on a search for new characters, in order to support our molecular findings.

Material and methods

Sample collection

Most specimens of *C. vittatus* and *C. sclopetarius* were obtained from the Crustacean Collection of the Department of Biology (CCDB) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP), University of São Paulo (USP). Other specimens were acquired through loan or donation from the National Crustacean Collection, Mexico City, Mexico (CNCR) and the Zoological Collection of the University of Louisiana, Lafayette, United States (ULLZ), or were collected by us or other researchers during the course of this study. Newly collected specimens were preserved directly in 80–90% ethanol, and their identifications were confirmed on the basis of morphological characters from available references (Holthuis 1959; Forest and Saint-Laurent 1968; Melo 1999; Nucci 2002).

Molecular analysis

The molecular analysis was based on the barcode region of the COI gene and a partial fragment of the 16S rDNA, which have been shown to be suitable for phylogenetic studies on decapods, clarifying the relationships among species (Schubart *et al*. 2000*a*; Tudge and Cunningham 2002; Mantelatto *et al*. 2006, 2007, 2009; Pileggi and Mantelatto 2010). All sequences of *C. vittatus* and *C. sclopetarius* used in this study were generated from our own extractions. Some additional comparative sequences obtained from other hermit crab species of the genus *Clibanarius* were included in order to make the analysis more consistent; two of them were retrieved from GenBank (Table 1). Genetic vouchers from which tissue was obtained have been deposited at CCDB/FFCLRP/USP under the accession numbers listed in Table 1.

DNA extraction, amplification and sequencing protocols followed Schubart *et al*. (2000*a*) with modifications according to Mantelatto *et al*. (2006, 2007, 2009), Pileggi and Mantelatto (2010) and Vergamini *et al*. (2011). Total genomic DNA was extracted from muscle tissue of the chelipeds, preferentially from the articulation between the carpus and merus. Muscle was ground and incubated for 1–12 h in 600 µL of lysis buffer and 200 µL of proteinase K (500 µg/µL) at 65°C; protein was separated by the addition of 200 µL of 7.5 M ammonium acetate before centrifugation. DNA was precipitated by the addition of 600 µL of cold absolute isopropanol, followed by centrifugation; the resultant pellet was washed with 70% ethanol, dried and resuspended in 10–20 µL of TE buffer.

An ~700-base-pair region of the COI gene was amplified from diluted DNA by means of polymerase chain reaction (PCR) in a Thermo (Portsmouth, NH, USA) PxE0.2ThermalCycler (thermal cycle: initial denaturing for 2 min at 94°C; annealing for 35 cycles: 30 s at 94°C, 30 s at 46−50°C, 1 min at 72°C; final extension 2 min at 72°C) using two pairs of primers: COH6 (5′-TADACTTCDGGRTGDCCAARAYCA-3′) and COL6b (5′-ACAAATCATAAAGATATYGG-3′) (Schubart and Huber 2006); LCO-1490 (5′-GGTCAACAAATCATAAAGA TATTG-3′) and HCO-2198 (5′−TAAACTTCAGGGTGACC AAAAAATCA-3′) (Folmer *et al*. 1994). A region with ~600 base pairs of the 16S rDNA gene was amplified (thermal cycle: initial denaturing for 5 min at 95°C; annealing for 38–42 cycles: 45 s at 95°C, 30 s at 48°C, 1 min at 72°C; final extension 3 min at 72°C) with the primers designated as follows: 16SH2 (5′-AGATAGAAACCAACCTGG-3′) and 16SL2 (5′-TGCCTG TTTATCAAAAACAT-3′) (for references on the primers see Schubart *et al*. 2000*a*, 2000*b*). PCR products were purified using the kit SureClean Plus and sequenced with the ABI Big-Dye Terminator Mix (Applied Biosystems, Carlsbad, CA, USA) in an

ABI Prism 3100 Genetic Analyzer (Applied Biosystems automated sequencer) following Applied Biosystems protocols. All sequences were confirmed by sequencing both strands. A consensus sequence for the two strands was edited and constructed with the aid of the computational program BIOEDIT 7.0.5 (Hall 2005).

Sequences were aligned using Clustal W (Thompson *et al*. 1994) with interface to BIOEDIT (Hall 2005) with default parameters. Ambiguous alignment regions were removed. Before the conclusion of the Maximum Likelihood (ML) analysis, sequences were submitted to a prior analysis in the software MODELTEST (Posada and Crandall 1998) to determine the model of sequence evolution that best fits the data, selected by the Akaike information criterion (Posada and Buckley 2004). The phylogenetic analysis was conducted using PAUP 4.0 β 10 (Swofford 2003) for Maximum Likelihood criterion with heuristic search and 100 random-addition sequence replications. The consistency of topologies was measured using a bootstrap method (1000 replicates), and only confidence values >50% were reported. Nucleotide composition, substitution frequencies, and pairwise distances were calculated with PAUP 4.0 β 10.

Morphological analysis

A search was made for diagnostic morphological differences to support our molecular results. This morphological analysis was conducted on the basis of the traditional taxonomic morphological characters gathered by reviewing the descriptions and diagnoses of *C. vittatus* and *C. sclopetarius* by Holthuis (1959), Forest and Saint-Laurent (1968), Sánchez and Campos (1978), Melo (1999) and Nucci (2002). We made an effort to find new characters that also could be used to support our molecular findings.

Results

Molecular analysis

The multiple sequence alignment for the COI gene had 679 positions for 39 specimens of *Clibanarius* (18 of *C. vittatus*, 16 of *C. sclopetarius*, and a single sequence of five other congener species included in the outgroup). The general-time reversible model of sequence evolution was the best-fitting model of substitution, with invariable sites and a gamma distribution (GTR+I+G) (Lanave *et al*. 1984; Rodríguez *et al*. 1990), and with the following parameters: assumed nucleotide frequencies

Table 1. Data for the hermit crab specimens used for the molecular analysis

Museum Collection Abbreviations: CCDB, Crustacean Collection of the Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo; ULLZ, Zoological Collection of University of Louisiana, Lafayette, USA

Species	Collection site, date	Catalogue number	GenBank accession numbers	
			16S rDNA	COI
C. albidigitus Nobili, 1901	Panama City, Panama, 2001	–	AF425323[A]	–
C. albidigitus Nobili, 1901	Punta Morales, Costa Rica, Sep. 2005	CCDB 1711	–	JN671591
C. antillensis Stimpson, 1859	Florida, USA, Jul. 1998	ULLZ 4683	DQ369941[B]	–
C. erythropus (Latreille, 1818)	Cadiz, Spain, Oct. 2009	CCDB 488	–	JN671592
C. lineatus (H. Milne Edwards, 1848)	Porosi, Nicaragua, Nov. 2001	CCDB 2444	–	JN671594
C. sclopetarius (Herbst, 1796)	Florianópolis, SC, Brazil, Apr. 2007	CCDB 2904	JN671520	–
C. sclopetarius (Herbst, 1796)	São Sebastião, SP, Brazil, May 2010	CCDB 2961	JN671523	JN671584, JN671585
C. sclopetarius (Herbst, 1796)	Guarapari, ES, Brazil, Nov. 2006	CCDB 2255	–	JN671582, JN671583
C. sclopetarius (Herbst, 1796)	Ilhéus, BA, Brazil, Jul. 2003	CCDB 2903	JN671519	–
C. sclopetarius (Herbst, 1796)	Ilhéus, BA, Brazil, Mar. 2009	CCDB 3070	–	JN671577, JN671578
C. sclopetarius (Herbst, 1796)	Maceió, AL, Brazil, Jan. 2005	CCDB 2949	JN671522	JN671575
C. sclopetarius (Herbst, 1796)	Tamandaré, PE, Brazil, Jun. 2010	CCDB 3062	JN671521	–
C. sclopetarius (Herbst, 1796)	Ipojuca, PE, Brazil, Oct. 2010	CCDB 3066	–	JN671573, JN671574
C. sclopetarius (Herbst, 1796)	Pirangi Parnamirim, RN, Brazil, Jun. 2011	CCDB 3361	–	JN671564, JN671565
C. sclopetarius (Herbst, 1796)	Fortaleza, CE, Brazil, May 2008	CCDB 2340	–	JN671562, JN671563
C. sclopetarius (Herbst, 1796)	Macapá Mangrove, PI, Brazil, Nov. 2004	CCDB 2902	–	JN671561
C. sclopetarius (Herbst, 1796)	Bocas del Toro, Panama, Aug. 2011	CCDB 3563	–	JQ 805893, JQ 805894
C. signatus Heller, 1861	Ilha Qeshm, Iran, Feb. 2006	CCDB 3694	–	JN671590
C. tricolor (Gibbes, 1850)	Cozumel, Mexico, Oct. 2010	CCDB 504	–	JN671593
C. vittatus (Bosc, 1802)	Florianópolis, SC, Brazil, Jul. 2003	CCDB 2946	–	JN671558, JN671559
C. vittatus (Bosc, 1802)	Florianópolis, SC, Brazil, Apr. 2007	CCDB 1889	JN671525	–
C. vittatus (Bosc, 1802)	Guaratuba, PR, Brazil, Feb. 2008	CCDB 2262	JN671528	JN671556, JN671557
C. vittatus (Bosc, 1802)	Ilha Comprida, SP, Brazil, Apr. 2011	CCDB 3363	–	JN671551, JN671552
C. vittatus (Bosc, 1802)	São Sebastião, SP, Brazil, Jun. 2002	CCDB 2947	–	JN671549
C. vittatus (Bosc, 1802)	Paraty, RJ, Brazil, Aug. 2007	CCDB 2237	JN671529	JN671548
C. vittatus (Bosc, 1802)	Ilhéus, BA, Brazil, Mar. 2009	CCDB 2907	JN671524	JN671545, JN671547
C. vittatus (Bosc, 1802)	Bragança, PA, Brazil, May 2010	CCDB 2944	–	JN671540, JN671541
C. vittatus (Bosc, 1802)	Mecoacan, TC, Mexico, Feb. 2011	CCDB 3364	–	JN671534, JN671535
C. vittatus (Bosc, 1802)	Florida, USA, Jul. 2001	CCDB 1189	JN671526	JN671530, JN671531
C. vittatus (Bosc, 1802)	Texas, USA, Sep. 2001	CCDB 1185	JN671527	JN671533

[A]S. D. Zaklan and C. Cunningham (unpubl. data).
[B]Mantelatto *et al*. (2006).

A = 0.3643, C = 0.1795, G = 0.1777, T = 0.2784; proportion of invariant sites I = 0.6060; variable sites followed a gamma distribution with shape parameter = 3.6340 (tree shown in Fig. 1).

The interspecific genetic variation found for the COI gene was 9.4–24.1%, and ranged from 9.4 to 11.9% between *C. vittatus* and *C. sclopetarius*. The intraspecific divergence ranged from 0.0 to 1.5% and from 0.0 to 5.8% for *C. sclopetarius* and *C. vittatus*. Concerning the intraspecific genetic divergence for *C. vittatus*, the highest values were found between specimens from Brazil and the Gulf of Mexico (4.7–5.8%) (Table 2).

A total of 572 positions of the 16S rDNA, excluding primer regions, were aligned for 13 specimens of *Clibanarius* (six specimens of *C. vittatus*, five specimens of *C. sclopetarius*, and two specimens of other species of the genus included in the outgroup). The optimal model of nucleotide substitution was the general-time-reversible model of sequence evolution (Lanave *et al*. 1984; Rodríguez *et al*. 1990) plus gamma-distributed rate heterogeneity (GTR+G), with the following parameters: assumed nucleotide frequencies A = 0.3444, C = 0.1941, G = 0.1163, T = 0.3452; proportion of invariant sites I = 0.0; variable sites followed a gamma distribution with shape parameter = 0.1607 (tree shown in Fig. 2).

The genetic divergence found among individuals of different species for the 16S rDNA gene ranged from 4.5 to 13.7%, and the genetic distance between *C. vittatus* and *C. sclopetarius* ranged from 4.5 to 5.9%. Thus, the genetic divergence measured between populations of *C. vittatus* and *C. sclopetarius* was in accord with the divergence found among other congeners at the interspecific level. Specimens of *C. sclopetarius* showed a null variation over the 572 nucleotides of the 16S rDNA gene that was studied. Comparing sequences of *C. vittatus* from Brazil and the Gulf of Mexico, we obtained 1.4% genetic divergence. However, considering separately specimens of *C. vittatus* from Brazil, or specimens of *C. vittatus* from the populations of the Gulf of Mexico, the sequences were identical in the 572 nucleotide regions of the 16S rDNA (Table 3).

Both trees constructed by means of Maximum Likelihood showed a clear separation between *C. vittatus* and *C. sclopetarius* (Figs 1, 2: Group I and II). In the COI phylogram, probably *Clibanarius lineatus* is a species closely related to *C. sclopetarius*

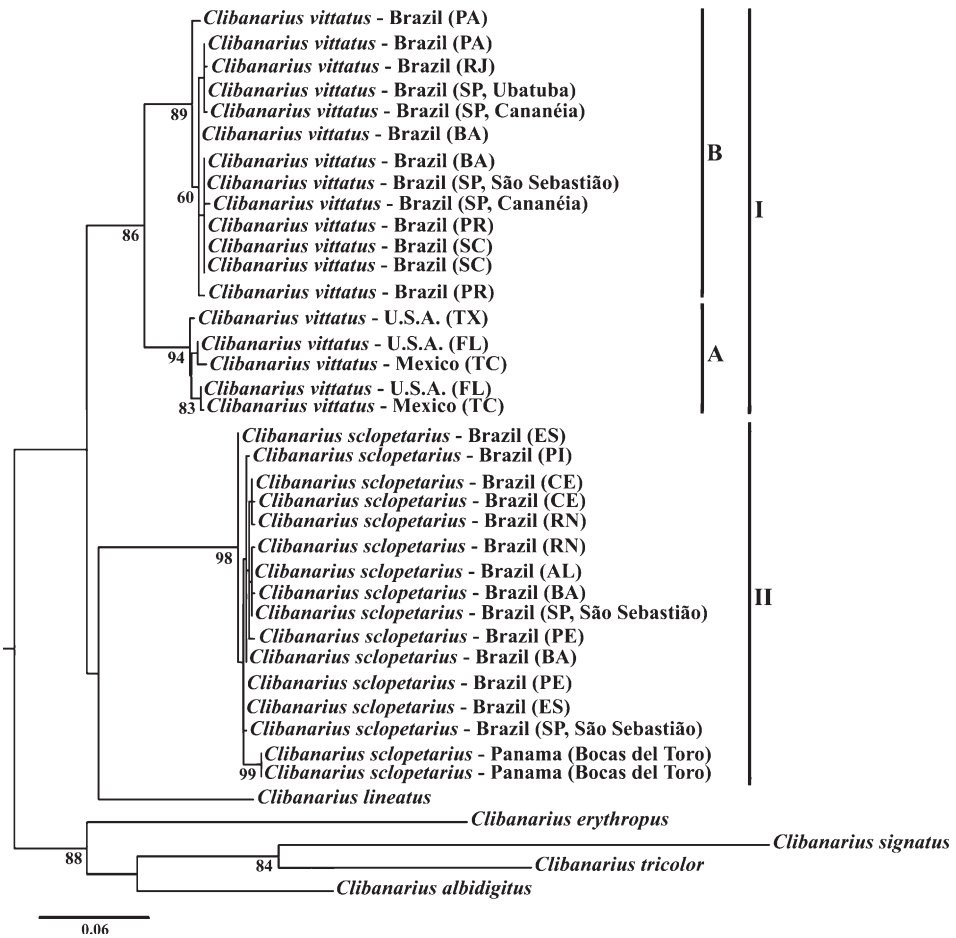

Fig. 1. Phylogram obtained from ML analysis of COI sequences for *C. albidigitus*, *C. erythropus*, *C. lineatus*, *C. signatus*, *C. sclopetarius*, *C. tricolor* and *C. vittatus*. Numbers are significance values for 1000 bootstraps; values ≤50% are shown only for the largest clades. Abbreviations: AL, Alagoas; BA, Bahia; CE, Ceará; ES, Espírito Santo; PA, Pará; PE, Pernambuco; PI, Piauí; PR, Paraná; RJ, Rio de Janeiro; RN, Rio Grande de Norte; SC, Santa Catarina; SP, São Paulo; FL, Florida; TX, Texas.

Table 2. Genetic divergence matrix of the partial sequences of the COI gene among various *Clibanarius* species

Specimens	1	2	3	4	5	6	7	8	9
1. *C. vittatus* (Gulf of Mexico)	0.000–0.013								
2. *C. vittatus* (Brazil)	0.047–0.058	0.000–0.015							
3. *C. sclopetarius* (Panama)	0.111–0.119	0.106–0.115	0.000						
4. *C. sclopetarius* (Brazil)	0.094–0.118	0.096–0.114	0.009–0.015	0.000–0.009					
5. *C. signatus* (Iran)	0.196–0.202	0.196–0.200	0.238–0.241	0.221–0.227	0.000				
6. *C. albidigitus* (Costa Rica)	0.152–0.158	0.160–0.168	0.169–0.170	0.168–0.177	0.209	0.000			
7. *C. erythropus* (Spain)	0.164–0.173	0.184–0.190	0.182–0.186	0.168–0.173	0.215	0.170	0.000		
8. *C. tricolor* (Mexico)	0.153–0.168	0.164–0.171	0.180–0.183	0.177–0.186	0.194	0.172	0.196	0.000	
9. *C. lineatus* (Nicaragua)	0.097–0.102	0.100–0.112	0.114–0.115	0.110–0.119	0.219	0.166	0.182	0.169	0.000

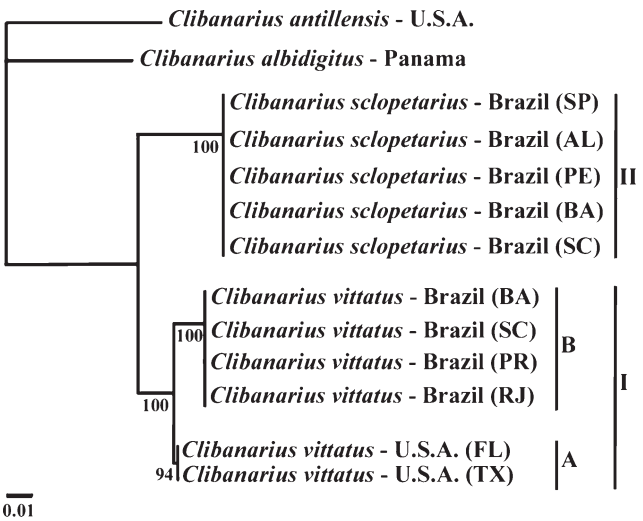

Fig. 2. Phylogram obtained from ML analysis of 16S rDNA sequences for *C. albidigitus*, *C. antillensis*, *C. sclopetarius* and *C. vittatus*. Numbers are significance values for 1000 bootstraps; values ≤50% are not shown. Abbreviations: FL, Florida; TX, Texas; BA, Bahia; SC, Santa Catarina; PE, Pernambuco; AL, Alagoas; SP, São Paulo; PR, Paraná; RJ, Rio de Janeiro.

Table 3. Genetic divergence matrix of the partial sequences of the 16S rDNA gene among various *Clibanarius* species

Specimens	1	2	3	4	5
1. *C. antillensis* (USA)	0.000				
2. *C. albidigitus* (Panamá)	0.107	0.000			
3. *C. sclopetarius* (Brazil) – 5 specimens	0.137	0.135	0.000		
4. *C. vittatus* (Brazil) – 4 specimens	0.135	0.118	0.059	0.000	
5. *C. vittatus* (Gulf of Mexico) – 2 specimens	0.135	0.114	0.045	0.014	0.000

and *C. vittatus*. However, as the group formed by these three species is not well-supported, it is not possible to infer the correct relationship among them.

Within Group II (*C. sclopetarius*), although specimens from Panama were placed in an isolated clade in the COI analysis, no structure at population level was evident. However, in Group I (*C. vittatus*) it was possible to identify two subgroups, with the constituents of each subgroup showing a low rate of genetic divergence. One subgroup comprised only individuals from the Gulf of Mexico (Figs 1, 2: Group A), and the other subgroup, only specimens from Brazil (Figs 1, 2: Group B).

Morphological assignments

Telson morphology, chela ornamentation, and the colour pattern of the eyestalks and of the last segment of the antennal peduncle, apart from the colour pattern of the walking legs, are the main morphological characters that we found to support our molecular findings (see more details in the Discussion).

Clibanarius sclopetarius (Herbst, 1796)

Material examined. 1 male, ULLZ 4657, Fort Pierce, S. Jetty of Inlet, Florida, USA; 1 male, CNCR 19989, Felipe Carrillo Puerto, Mexico; 1 male, CNCR 19217, Felipe Carrillo Puerto, Mexico; 2 males and 2 females, CCDB 3563 (DNA voucher), Smithsonian Station, Bocas del Toro, Panama; 1 male and 1 female, CCDB 2902 (DNA voucher), Macapá Mangrove, Piauí, Brazil; 2 males, CCDB 2340 (DNA voucher), Meireles Beach, Fortaleza, Ceará, Brazil; 2 males, CCDB 3066 (DNA voucher), Muro Alto, Ipojuca, Pernambuco; 5 males, CCDB 3062 (DNA voucher), Carneiros Beach, Tamandaré, Pernambuco, Brazil; 1 male, CCDB 2949 (DNA voucher), Gunga Beach, Maceió, Alagoas, Brazil; 3 males, CCDB 3070 (DNA voucher), Backdoor Beach, Ilhéus, Bahia, Brazil; 2 males, CCDB 2255 (DNA voucher), Guarapari Channel, Guarapari, Espírito Santo, Brazil; 2 males, CCDB 2722, and 7 males, CCDB 2961 (DNA voucher), Araçá Mangrove, São Sebastião, São Paulo, Brazil.

Clibanarius vittatus (Bosc, 1802)

Material examined. 4 males, CCDB 3783, Indian River Lagoon, Florida, USA; 4 males, CCDB 1189 (DNA voucher), Choctawhatchee Bay, Florida, USA; 3 males, CCDB 3695, Eagle Harbor, Florida, USA; 4 males, CCDB 3364 (DNA voucher), Mecoacán Lagoon, Mecoacán, Tabasco, Mexico; 5 males, CCDB 2944 (DNA voucher), Urujuçuaba Beach, Bragança, Pará, Brazil; 1 male, CCDB 2905, Macapá Mangrove, Macapá, Piauí, Brazil; 1 male, CCDB 2907 (DNA voucher), Maramata Beach, Ilhéus, Bahia, Brazil; 1 male, CCDB 2237 (DNA voucher), Pontal Beach, Paraty, Rio de Janeiro, Brazil; 1 male, CCDB 1651, Itaguá Beach, Ubatuba, São Paulo, Brazil; 4 males, CCDB 2947 (DNA voucher), Araçá Mangrove, São Sebastião, São Paulo, Brazil; 5 males, CCDB 3363 (DNA voucher), Trincheira Beach, Ilha Comprida, São Paulo, Brazil;

2 males, CCDB 2277, Brava Beach, Guaratuba, São Paulo, Brazil; 1 male, CCDB 1889 (DNA voucher), Sambaqui Beach, Florianópolis, Santa Catarina, Brazil.

Discussion

In contrast to the previous hypothesis on the synonymy between *C. vittatus* and *C. sclopetarius*, the present investigation, based on molecular analysis of partial sequences of 16S rDNA and barcoding COI, supported the maintenance of *C. vittatus* and *C. sclopetarius* as distinct taxonomic species, and this result was also supported by the morphological analysis.

Three important results obtained here by the molecular analysis justify the separation of the two species. (1) The interspecific genetic divergence is greater than the intraspecific molecular variation for both genes. If the genetic variation between *C. vittatus* and *C. sclopetarius* were less than the intraspecific variation of each species, it could be an indication that these two species are synonymous. However, that was not observed in our results, so we considered that this to be an evidence for the non-synonymisation of the two species. (2) The placement of geographically close specimens of *C. vittatus* and *C. sclopetarius* in two distinct groups (Figs 1, 2 – Group I and II) indicates that the separation of these two groups is due to interspecific variation between the two questionable species, rather than to intraspecific variation among the samples from different populations. This occurred, for example, in the case of specimens of *C. vittatus* and *C. sclopetarius* from Santa Catarina and Bahia (Brazil). (3) The tree generated with the sequences of the barcoding COI gene (Fig. 1) shows that another species, *C. lineatus*, may be more closely related to *C. sclopetarius* or *C. vittatus* than the latter are to each other. However, to achieve a more precise elucidation of the phylogenetic relationships among the species of *Clibanarius*, a more complete analysis must be developed, including other species of this genus and additional molecular markers.

In addition to the colour pattern of the walking legs (Figs 3*A*, 4*A*), the coloration of the eyestalks (characterised by the presence

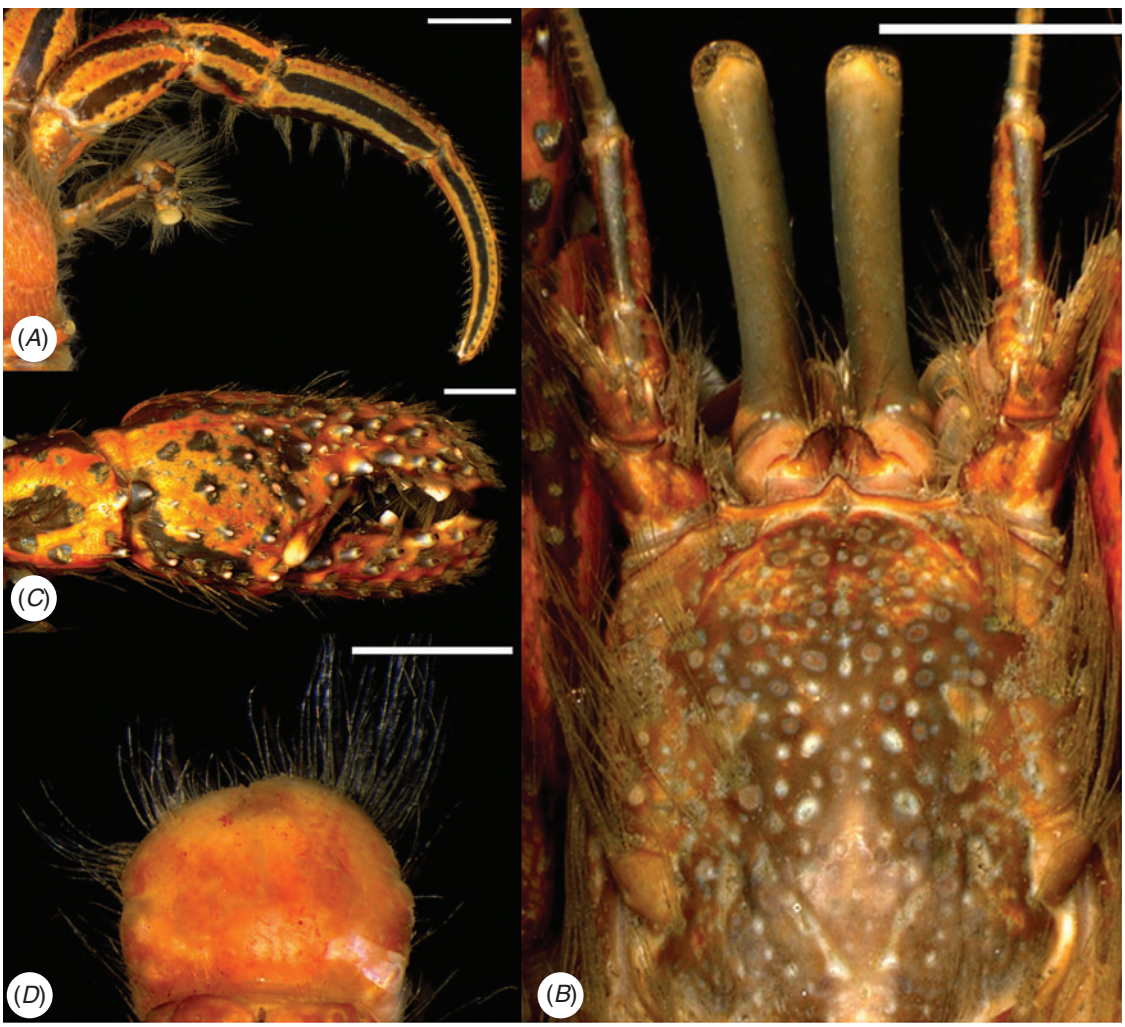

Fig. 3. Male specimen of *Clibanarius sclopetarius* from Araçá Mangrove, São Sebastião, São Paulo, Brazil (CCDB 2961). (*A*) External lateral view of third right pereiopod; (*B*) dorsal view of cephalothoracic shield, eyestalks and antennal peduncles; (*C*) dorsal view of left chela; (*D*) dorsal view of telson. Scale: *A*, 5 mm; *B*, 5 mm; *C*, 2 mm; *D*, 2 mm.

of an olive-green stripe on the dorsal inner surface of *C. vittatus*) and the coloration of the last segment of the antennal peduncle (characterised by the presence of an olive-green stripe on the dorsal margin of *C. sclopetarius* and a light-coloured stripe on the dorsal margin in *C. vittatus*) (Figs 3*B*, 4*B*) contribute to the questionable separation of these two species. The relevance of using coloration as a character to support phylogenies is questionable because of its high variability. However, coloration has proved to be of systematic importance for several species of decapod crustaceans (Bruce 1975; Knowlton 1986; Knowlton and Mills 1992; Sarver *et al*. 1998; Macpherson and Machordom 2001; Hiller *et al*. 2006), more specifically for hermit crabs (Ball and Haig 1974; Malay and Paulay 2010). Although the biological function is still poorly understood, one hypothesis suggests that coloration has played an important role in the process of sexual selection (Hiller *et al*. 2006). Therefore, considering that the species in question live in sympatry, the specific coloration may play a role in conspecific recognition, thus favouring the formation of monospecific assemblages that aid in locating mating partners (Hiller *et al*. 2006).

Furthermore, the distribution and quantity of chela tubercles and spines supported the separation of these two species. These ornamentations are concentrated on the dorsal surface, and are less abundant in *C. sclopetarius* than in *C. vittatus* (Figs 3*C*, 4*C*). Another important character to distinguish *C. vittatus* and *C. sclopetarius* and to support the molecular findings was the telson morphology (Figs 3*D*, 4*D*). The distal lobes were more rounded in *C. vittatus*, and the median cleft of the terminal margin was deeper in *C. vittatus* than in *C. sclopetarius*, as were the clefts of the lateral margins.

Larval taxonomy

Some studies on the larval development of species of *Clibanarius* provide additional support for our results. Despite the general homogeneity in larval morphology in *Clibanarius*, some

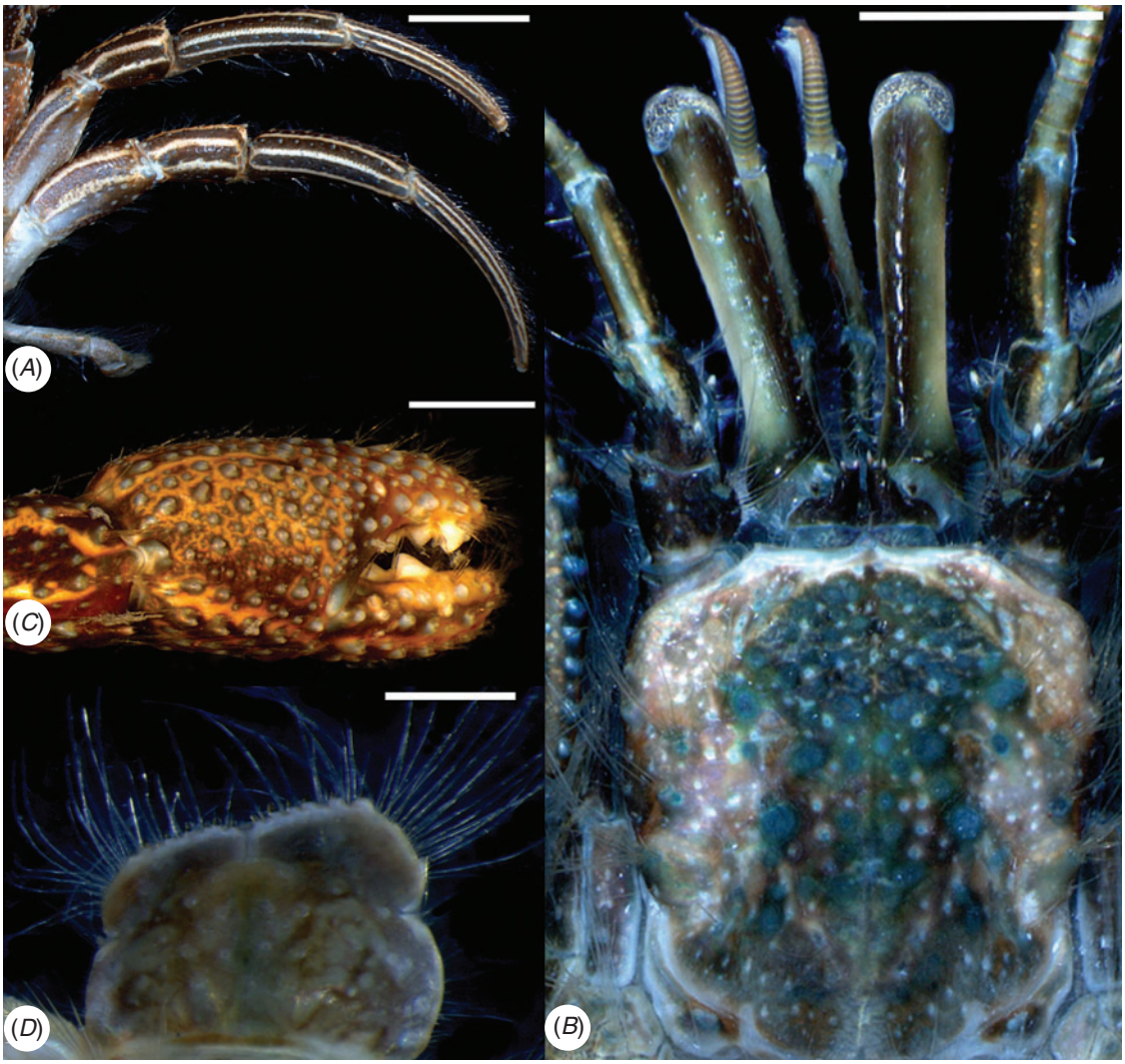

Fig. 4. Male specimen of *Clibanarius vittatus* from Trincheira Beach, Cananéia, São Paulo, Brazil (CCDB 3363). (*A*) External lateral view of second and third right pereiopod; (*B*) dorsal view of cephalothoracic shield, eyestalks and antennal peduncles; (*C*) dorsal view of left chela; (*D*) dorsal view of telson. Scale: *A*, 5 mm; *B*, 5 mm; *C*, 2 mm; *D*, 1 mm.

variations exist among congeneric species (Bartilotti *et al*. 2008). For *C. vittatus* and *C. sclopetarius*, these variations are related to differences in the morphology of the telson process beyond the second larval stage, and to the number of zoeal stages (Lang and Young 1977; Brossi-Garcia 1987; Siddiqui *et al*. 1991; Bartilotti *et al*. 2008). Concerning the fourth telson process as a fused spine, beyond the second larval stage, this spine is well developed in *C. vittatus* (Lang and Young 1977) and relatively small in *C. sclopetarius* (Brossi-Garcia 1987). The importance of this difference is sustained by some studies on anomuran larvae that have considered the telson process to be a very important diagnostic character (McLaughlin *et al*. 1992, 1993). Siddiqui *et al*. (1991) reported the occurrence of four or five zoeal stages in *Clibanarius* and noted that the fifth zoeal stage is necessary for *C. vittatus* to complete its larval development. Likewise, Lang and Young (1977) also showed that five zoeal stages are commonly involved in the developmental series of *C. vittatus*, with the absence of a well developed mandibular palp distinguishing the fourth from the fifth stage. For *C. sclopetarius*, the fifth stage might be unnecessary to complete its developmental series. The larval sequence of *C. sclopetarius* generally includes four zoeal stages (Brossi-Garcia 1987). Bartilotti *et al*. (2008) speculated that the presence of a fifth stage may result from a laboratory artefact, due to the morphological similarity between the fourth and the fifth zoeal stages. The appearance of an extra larval stage may be a consequence of the use of inappropriate rearing conditions (Gore 1985) or a case of intraspecific variation (Ajmal Khan and Natarajan 1981).

Spermiotaxonomy

The two species share a very similar spermatophore structure (Hess and Bauer 2002; Santos and Mantelatto 2011), with conspicuous differences that provide support for our previous molecular and morphological findings. The species comprising the superfamily Paguroidea (Anomura) have a complex spermatophore with a tripartite structure consisting of an ampulla, a stalk and a foot (Krol *et al*. 1992). However, both species, *C. vittatus* and *C. sclopetarius*, represent exceptions to this character, due to the presence of non-pedunculate spermatophores (Hess and Bauer 2002; Santos and Mantelatto 2011). The non-tripartite pattern was also noted for another congener, *C. longitarsus* (De Haan, 1849) (Uma and Subramoniam 1984). On the basis of this character, Santos and Mantelatto (2011) proposed that these three species should be placed together in a closer phylogenetic relationship than with other species whose spermatophores are pedunculate: *Clibanarius erythropus* (Latreille, 1818) (Tirelli *et al*. 2007), *Clibanarius misanthropus* (Risso, 1826) (Mouchet 1931), *Clibanarius virescens* (Krauss, 1843) (Tudge 1991) and *Clibanarius corallinus* (Milne Edwards, 1848) (Tudge 1991). Unfortunately, we lack available material of all these species to perform a molecular analysis and check this assertion.

Biogeographical variability

Finally, our molecular data on global-scale surveys of sequence diversity showed a considerable difference between *C. vittatus* from Brazil and from the Gulf of Mexico, which were placed in two separate clades that were well supported by high bootstrap values. This biogeographical pattern of molecular variation is corroborated by some subtle morphological divergences (M. Negri and F. L. Mantelatto, unpubl. data). The most obvious morphological variations observed were in the colour pattern of the pereiopods (M. Negri and F. L. Mantelatto, unpubl. data). According to the description and diagnosis of *C. vittatus*, the carpus of the walking legs has two light stripes on the external lateral surface (Holthuis 1959; Forest and Saint-Laurent 1968; Melo 1999; Nucci 2002). However, whereas the specimens from Brazil follow this description, the carpus of specimens from North America shows three light stripes on the external lateral surface; apart from that, *C. vittatus* from North America has pale patches surrounding the bases of some tubercles and spines of its chela (M. Negri and F. L. Mantelatto, unpubl. data). Moreover, Forest and Saint-Laurent (1968) mentioned the small size of the spines and tubercles in the chela of *C. vittatus*, but a morphological analysis showed this pattern of chela ornamentation only for specimens from Brazil and not for specimens from North America, whose spines and tubercles are similar to those of *C. sclopetarius* (M. Negri and F. L. Mantelatto, unpubl. data).

On the basis of the above morphological differences, separation of *C. vittatus* from North and South America into two distinct species seems appropriate (M. Negri and F. L. Mantelatto, unpubl. data). Additionally, given our molecular results, the hypothesis of two cryptic species designated as *C. vittatus* is even more strongly supported. The 16S rDNA and mainly the COI gene showed considerable genetic divergence between these two groups.

To develop a robust examination of the evolutionary story of these two species (*C. vittatus* and the other one), samples from intermediate geographical areas between the North and South Atlantic must be included in the analysis. Unfortunately, no known collections have been attempted in this region, so the complete morphological description of specimens from these areas is not known. The descriptions and illustrations of *C. vittatus* present in Holthuis (1959) and Sánchez and Campos (1978) indicate that the specimens from Suriname and Colombia are similar to those from Brazil.

Unlike the findings for *C. vittatus*, a biogeographical genetic structure was not determined for *C. sclopetarius*. This reflects the low rate of molecular divergence among individuals of these species, and the genes (16S rDNA and COI) are not sufficiently variable for any population structure (Vergamini *et al*. 2011) to be evident. Only specimens of *C. sclopetarius* from populations in South and Central America were included in the molecular studies. Nevertheless, the surveys of the morphology of *C. sclopetarius*, covering a wider latitudinal distribution, showed no significant variation.

In sum, we find no genetic or morphological justification for the synonymy of *C. sclopetarius* and *C. vittatus*. These findings are also supported by larval characters (Lang and Young 1977; Brossi-Garcia 1987; Siddiqui *et al*. 1991; Bartilotti *et al*. 2008). Moreover, the phylogenetic analysis using COI and 16S rDNA revealed a significant genetic divergence between two clades of *C. vittatus* (Figs 1, 2, Group A – specimens from the Gulf of Mexico, and Group B – South American specimens). This result, in combination with morphological data (M. Negri and

F. L. Mantelatto, unpubl. data), contribute to supporting the proposition that these two clades constitute different species.

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