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Original article

Phylogenetic position of two Patagonian Cibicididae (Rotaliida, Foraminifera): *Cibicidoides dispars* (d'Orbigny, 1839) and *Cibicidoides variabilis* (d'Orbigny, 1826)

Position phylogénétique de deux Cibicididae (Rotaliida, Foraminifera) patagoniens : Cibicidoides dispars (d'Orbigny, 1839) et Cibicidoides variabilis (d'Orbigny, 1826)

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Abstract

Cibicidoides dispars and *Cibicidoides variabilis* are two neritic cibicidids commonly found on the Patagonian coasts. Phylogenetic analyses of partial SSU rDNA sequences show that they both belong to the genus *Cibicidoides*. *Cibicidoides dispars* branches close to *Cibicidoides wuellerstorfi*, whereas *C. variabilis* clusters with *Cibicidoides pachyderma*. In both cases, species clustering together are well separated morphologically and ecologically but close genetically. Molecular data indicate clearly that *C. dispars* and *C. variabilis* are well separated genetically from *Cibicidoides lobatulus*, another shallow water cibicidid sharing a similar ecology and morphology. Moreover, our molecular results show that neritic and bathyal or abyssal species are found together in different clades, suggesting multiple colonization events from shallow to deep water or vice versa. The analysis of more variable ITS rDNA region, on the other hand, reveals small differences between individuals of *C. variabilis* sampled in the south and north of Chilean Patagonia, which could indicate a cryptic speciation undergoing in this species.

Keywords: Benthic foraminifers; Molecular phylogeny; Patagonia; Cibicididae; SSU and ITS rDNA

Résumé

Cibicidoides dispars et *Cibicidoides variabilis* sont des espèces néritiques communes des côtes patagoniennes. L'analyse phylogénétique de séquences partielles du gène de la petite sous-unité (18S) de l'ARN ribosomique (ARNr) montre que ces deux espèces appartiennent au genre *Cibicidoides*. *Cibicidoides dispars* est étroitement apparenté à *Cibicidoides wuellerstorfi*, tandis que *C. variabilis* est proche de *Cibicidoides pachyderma*. Dans les deux cas, les espèces appartenant à un même clade sont bien séparées morphologiquement et écologiquement mais proches génétiquement. Les données moléculaires indiquent aussi clairement que *C. dispars* et *C. variabilis* sont bien différents génétiquement de *Cibicidoides lobatulus*, une autre espèce néritique partageant une écologie et une morphologie similaires. Nos résultats moléculaires montrent en outre que des espèces néritiques et bathyales ou abyssales se retrouvent ensemble dans différents clades, ce qui pourrait suggérer des événements de colonisation multiples d'eau peu profonde à profonde ou inversement. D'autre part, l'analyse d'une région plus variable de l'ADN ribosomique (ADNr), l'espaceur intragénique transcrit (ITS en anglais), révèle de petites différences entre les spécimens de *C. variabilis* échantillonnés au sud et au nord de la Patagonie chilienne, ce qui pourrait indiquer une spéciation cryptique dans cette espèce.

Mots clés : Foraminifères benthiques ; Phylogénie moléculaire ; Patagonie ; Cibicididae ; Petite sous-unité et espaceur intragénique transcrit de l'ADNr

1. Introduction

Alcide d'Orbigny (1839a) was the first scientist to study shallow water Patagonian foraminifers. In the

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20th century, Heron-Allen and Earland (1932) studied foraminifers from the Falkland area and the adjacent coast of Argentina. Later, the number of foraminiferal studies involving Patagonia increased, focusing on south Chile (Figueroa et al., 2005; Hromic, 1996b; Hromic et al., 2006; Ishman and Martinez, 1995; Zapata and Moyano, 1997; Zapata et al., 1995), Tierra del Fuego (Lena, 1996) and the strait of Magellan (Hromic, 1996a, 1997; Marchant, 1993; Violanti et al., 2000; Zapata et al., 1988).

Cibicidoides dispars was originally described in Falkland Islands samples as *Truncatulina dispars* and its test was characterized by a significant difference in porosity between the umbilical (small pores) and the spiral (large pores) sides (d'Orbigny, 1839a). Heron-Allen and Earland (1932) found *C. dispars* widely distributed around the Falkland Islands and also near Cape Horn, but believed it to be a variant of *Cibicidoides lobatulus* (Walker and Jacob), 1798, *Cibicidoides dispars* has also been reported as *Cibicides dispars* from south Brazil (Duleba et al., 1999), Argentina (Boltovskoy et al., 1980; Gómez et al., 2005), Chile (Figueroa et al., 2005; Hromic et al., 2006) and New Zealand (Hayward et al., 2002). This morphospecies is thought to live at shelf to upper bathyal depths in temperate waters with moderately low food supply, attached to substrates in high energetic environments (Boltovskoy et al., 1980; Hayward et al., 2002, 2003; Hromic, 2006).

Cibicidoides variabilis was also described by d'Orbigny in 1826, as *Truncatulina variabilis*. Its coiling is regular in the first whorl (juvenile stages), but becomes completely chaotic during the later growth stages with coarse porosity on both sides (d'Orbigny, 1826). Although the type locality of this morphospecies is the Mediterranean, its distribution appears cosmopolitan. Reported under several generic names which include *Cibicides*, *Cibicidella*, *Cibicidinella*, *Dyocibicides* and *Truncatulina*, it has been found off the Canary Islands (d'Orbigny, 1839b), Guam (Richardson and Clayshulte, 2003), New Zealand (Chapman, 1909), Falkland Islands (Heron-Allen and Earland, 1932) and Patagonia (Boltovskoy et al., 1980; Figueroa et al., 2005; Hromic et al., 2006).

Previous molecular studies based on SSU rDNA sequences (Schweizer et al., 2008, 2009) have shown that cibicidids (*Cibicides* and *Cibicidoides*) are closely related to *Melonis* and *Hanzawaia* and belong to rotaliid clade 3, which also includes the genera *Chilostomella*, *Oridorsalis*, *Pullenia*, *Epistominella*, *Stainforthia*, *Bulimina* and *Nonionella*. In phylogenetic analyses, the cibicidids form two separate clades, which are not distinguished on the basis of the test shape (planoconvex/biconvex). One clade corresponds to the genus *Cibicides* and includes *Cibicides refulgens* and *Cibicides* sp., a species from Antarctica, while the other clade corresponds to the genus *Cibicidoides* and comprises *Cibicidoides pachyderma*, *Cibicidoides ungerianus*, *Cibicidoides wuellerstorfi* and *C. lobatulus* (Schweizer et al., 2009).

Here we investigate the phylogenetic position and the morphology of the Patagonian shallow water *C. variabilis* and *C. dispars*. We establish the position of both morphospecies within Cibicididae and their relationships with other *Cibicidoides* based on the SSU rDNA sequences. We also examine the intraspecific variability of *C. variabilis* by analysing the sequences of ITS (internal transcribed spacer) rDNA region from specimens coming from two different locations, separated by about 1300 km.

2. Material and methods

2.1. Sample collection and DNA extraction

The foraminifers were sampled at two sites in Chilean Patagonia (Fig. 1). Seno Otway (52°33'39"S/71°43'54"W) was investigated in January 2006. This site is composed of two large inner bays with relatively low salinity values, connected to the Strait of Magellan by fjords. The sampled site was a pebble beach where foraminifers were collected at low tide by hand (Fig. 1a). The second site, Punta Huinay (42°22'30"S/72°25'38"W), was sampled in March 2006 (Fig. 1b). This site is situated at the termination of the Comau Fjord which has steep rocky slopes covered with seaweeds. Foraminifers were collected from the seaweeds by SCUBA diving to a depth of 9.5 m, below the fresh water layer. Collected sediment, rocks and seaweeds were immediately rinsed and sieved on site with seawater and maintained at collection site temperatures (~10 °C). Samples were

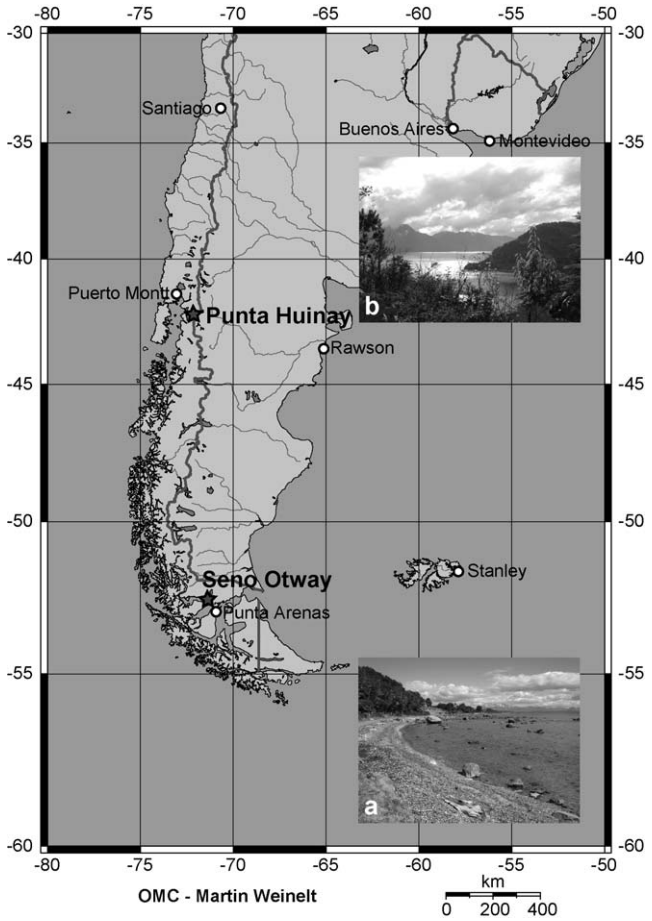

Fig. 1. Map of the south of South America with the localization of the two sampled sites. Pictures of landscapes at Seno Otway (**a**) and at Punta Huinay (**b**).

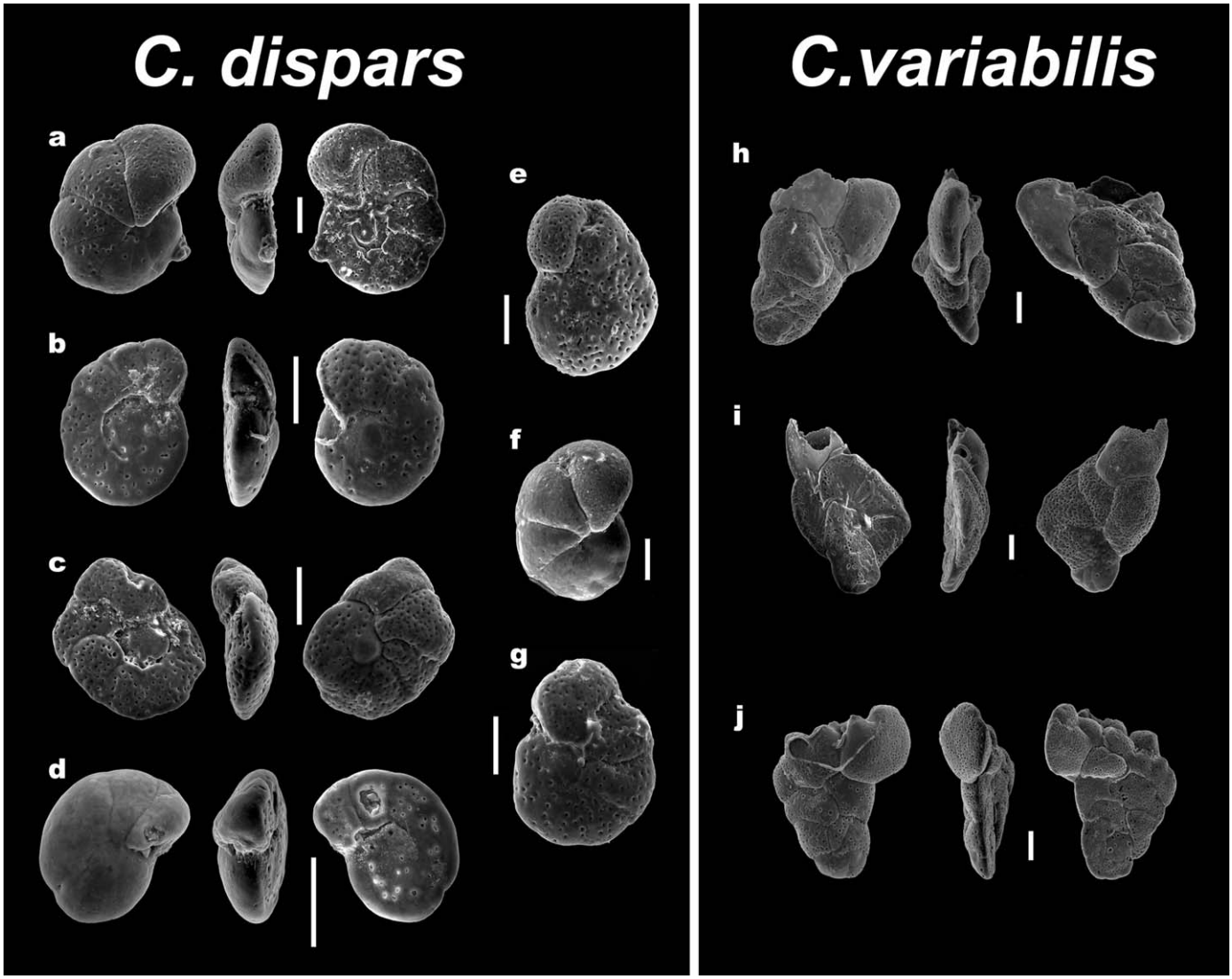

Fig. 2. SEM pictures of *C. dispars* (**a**–**g**) and *C. variabilis* (**h**–**j**). Scale bar = 100 μm.

processed within one week of collection. Live specimens were recognized by cytoplasm colour, test condition, the accretion of detritus near the aperture, the presence of reticulopods or the movements of the test. Once identified, live individuals were cleaned with a brush in filtered seawater and picked for DNA extraction in a guanidine buffer (Pawlowski, 2000). Other specimens were dried on Chapman slides and some individuals were imaged using a scanning electron microscope (SEM) at the ETH in Zurich (Fig. 2).

2.2. PCR amplification, cloning, and sequencing

In the first analysis, two regions of SSU rDNA were examined. Both regions were comprised of approximately 1000 nucleotides (nt) each and were situated at the 5' end (fragment sA-s6) and the 3' end (fragment s14-sB) respectively. The second analysis (∼1000 nt in total) included the rDNA region 5.8S and the ITS regions 1 and 2. The SSU fragments were amplified using the primers described in Schweizer et al. (2008) and the ITS region with primers described in Schweizer et al. (2005). The PCR reactions were performed with 2 μL of DNA template in a total volume of 50 μL with 40 cycles for the amplification (30 s at 94 °C, 30 s at 50 °C and 120 s at 72 °C), followed by 5 min at 72 °C for final extension. Semi-nested PCR were carried out with 2 μL of amplified products, using 35 cycles with 30 s at 52 °C instead of 50 °C, all other parameters remaining unchanged. Positive PCR products were purified using High Pure PCR Purification Kit (Roche Diagnostics). Most of the SSU PCR products were sequenced directly, while the remaining PCR products (samples 6200, 6201 and 6592 for the SSU and all ITS products) were ligated in the pGEM-T Vector (Promega) and cloned using ultracompetent cells XL2-Blue MRF' (Stratagene). Sequencing reactions were prepared using ABI-PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit and analysed with an ABI-PRISM 3100 DNA sequencer (Applied Biosystems), all according to the manufacturer's instructions. The new sequences have been deposited in the EMBL/GenBank database (Table 1).

Table 1
List of studied Patagonian cibicidids with their DNA numbers and the EMBL/GenBank accession numbers for their SSU (fragments sA-s6 and s14-sB) and ITS rDNA sequences.

DNA number	Species	Sampling site	Number of specimens	Accession numbers		
				sA-s6	s14-sB	ITS
6200	*C. variabilis*	Seno Otway	1		GU113132–GU113134	GU113093–GU113100
6201	*C. variabilis*	Seno Otway	1		GU113135, GU113136	
6205	*C. variabilis*	Seno Otway	1	GU113114	GU113125	GU113101–GU113107
6206	*C. variabilis*	Seno Otway	3		GU113129	
6473	*C. dispars*	Punta Huinay	1	GU113108	GU113119	
6474	*C. dispars*	Punta Huinay	1	GU113110	GU113121	
6476	*C. dispars*	Punta Huinay	1	GU113112	GU113123	
6479	*C. dispars*	Punta Huinay	1	GU113113	GU113124	
6480	*C. dispars*	Punta Huinay	1	GU113109	GU113120	
6481	*C. dispars*	Punta Huinay	1		GU113118	
6483	*C. dispars*	Punta Huinay	3	GU113111	GU113122	
6484	*C. variabilis*[a]	Punta Huinay	3	GU113116	GU113127	GU113086–GU113091
6485	*C. variabilis*[a]	Punta Huinay	5	GU113115	GU113126	
6486	*C. variabilis*[a]	Punta Huinay	5	GU113117	GU113128	GU113092
6592	*C. variabilis*	Punta Huinay	1		GU113130, GU113131	

[a] Specimens identified morphologically as *C. dispars*.

2.3. Phylogenetic analysis

Sequences of both SSU rDNA fragments (sA-s6 and s14-sB) were concatenated and aligned manually together (2455 sites) with other cibicidids' sequences using Seaview (Galtier et al., 1996). The unalignable regions were removed, leaving 1085 sites (number of sites calculated with Phylo_Win, Galtier et al., 1996) for use in phylogenetic analyses. Maximum likelihood (ML) analyses were performed with 100 bootstrap (BS) replicates by PhyML 2.4.4 (Guindon and Gascuel, 2003). Bayesian analyses (BA) were calculated with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001). Two independent analyses were done at the same time with four simultaneous chains run for 3'000'000 generations, and sampled every 100 generations with 7'500 initial trees discarded as burn-in after convergence was reached. The posterior probabilities (PP), calculated during the BA, estimated the reliability of internal branches. The General Time Reversible (GTR) model (Lanave et al., 1984; Rodriguez et al., 1990), which was selected previously for Cibicididae (Schweizer et al., 2009), was also chosen as the evolutionary model here. To correct for among-site rate variations, the proportion of invariable sites (I) and the alpha parameter of gamma distribution (Γ), with six rate categories, were calculated by PhyML and MrBayes (GTR + I + Γ).

ITS sequences were aligned separately and 789 out of 1283 sites were retained for the analysis. Phylo_Win was used to build a BIONJ tree (Gascuel, 1997) under the Kimura's two parameters (K2P) model (Kimura, 1980) with 1'000 BS replicates.

3. Results and discussion

3.1. Morphological description of C. dispars

Cibicidoides dispars (Fig. 2a–g) has a trochospiral and planoconvex test with a flatter spiral side serving as an attachment surface. The umbilical side is slightly convex but never very high, presenting a rather flat profile. The test has an oval to subcircular outline and is relatively small compared to other shallow water cibicidids, with a maximal diameter of 180–580 μm in our sample. The most recent sutures are depressed whereas the older ones tend to be more flush. The periphery is rounded and the chambers are smooth to slightly lobate. According to d'Orbigny (1839a), the difference in porosity between opposite sides is the main distinctive criterion to identify the species. However, Boltovskoy et al. (1980) observed specimens with equal porosity on both sides. In our samples we also found specimens with a more porous spiral side (Fig. 2a, d) and others equally porous on both sides (Fig. 2b, c). As in other cibicidids, the aperture is a curved slit bordered with a lip and situated at the peripheral margin with greater development on the umbilical side. All our specimens exhibited an umbilical knob (Fig. 2a–g), a feature which is not always present in other *C. dispars* (Boltovskoy et al., 1980).

3.2. Morphological description of C. variabilis

In the early stages of development, the test of *C. variabilis* is trochospiral and planoconvex with a regular addition of chambers, similar to other cibicidids. After the first whorl, chambers are added more chaotically, usually continuing along the same growth axis with peripheral addition. The direction of growth often changes from one chamber to the next (Fig. 2h–j). The test is relatively large compared to other cibicidids, with a maximal diameter of 617–767 μm in our sample. The spiral side is flat to concave, whereas the umbilical side is slightly convex. The periphery of the mature test is rounded, the sutures are depressed and the chambers slightly lobate. The pores are coarse on both sides. As in other cibicidids, the aperture is a curved slit bordered with a lip and situated at the peripheral margin. However, if the chamber is situated on another growth axis than the principal

one, the aperture may develop in another region of the chamber (Fig. 2 h, j).

3.3. Molecular data

3.3.1. SSU rDNA sequences

Nineteen new sequences of Patagonian *C. dispars* and *C. variabilis* were obtained for the fragment s14-sB and 10 for the fragment sA-s6 (Table 1). These sequences were aligned with 45 SSU rDNA sequences belonging to four morphospecies of *Cibicidoides* (*C. lobatulus*, *C. pachyderma*, *C. ungerianus*, *C. wuellerstorfi*). *Cibicidoides refulgens* and *Cibicides* sp. were used as an outgroup. Sequences published by other authors (Blümel et al., unpublished data; Pawlowski et al., 2007) are given with their EMBL/GenBank accession number. Maximum likelihood analyses of the partial SSU rDNA sequences produced a tree (Fig. 3a) consistent with the one in Schweizer et al. (2009), although statistical support is significantly lower in some places due to the reduced number of sites analysed. The topology of the Bayesian tree differs only by a lack of resolution in the clade *C. pachyderma* + *C. variabilis* (Fig. 3b).

Patagonian specimens of *C. dispars* and *C. variabilis* group clearly inside the clade of *Cibicidoides* (Fig. 3a), as previously defined (Schweizer et al., 2009). Therefore, the generic name *Cibicidoides* should be used for both these species and not *Cibicides* (Boltovskoy and Theyer, 1970; Hayward et al., 2002; Hromic, 2007; Marchant, 1993) or *Cibicidella* (Langer and Schmidt-Sinns, 2006).

Most of the *C. dispars* sequences form a well-defined clade, with 100% BS/1.00 PP support, branching within the group of *C. ungerianus* and *C. wuellerstorfi* (Fig. 3a), as a sister-clade to *C. wuellerstorfi* and two undetermined *Cibicidoides* (5227 and 2524). Three specimens identified morphologically as *C. dispars* (6484, 6485, 6486) were found grouping together with *C. variabilis*. This misidentification could have been due to the fact that juvenile *C. variabilis* have a regular growth contrary to older individuals (d'Orbigny, 1826).

Despite a low divergence (around 1%) characterizing the clade *C. wuellerstorfi* + *Cibicidoides* sp. + *C. ungerianus* (Schweizer et al., 2009), the *C. dispars* sequences clearly differ from the others by a characteristic insertion in the 37/f region, as defined by Pawlowski and Lecroq (2010). They also present minor variations in other regions, for example in the region 45/e (Pawlowski and Lecroq, 2010), which is usually well conserved in *Cibicidoides*, except *C. lobatulus* (Schweizer et al., 2009). Nevertheless, the position of *C. dispars* as the sister group of *Cibicidoides* sp. 2524, *Cibicidoides* sp. 5227 and *C. wuellerstorfi* is not well supported in the SSU tree (Fig. 3a), and more variable region will be necessary to resolve the relationships between these species.

In all our analyses, the sequences of *C. variabilis* branch together with those of *C. pachyderma* and *Cibicidoides* sp. 5305. However, despite evident morphological and ecological differences, the divergence between these species is very low (< 1%). In consequence, the relationships within this clade are not resolved with very low BS values in the ML analysis (20% BS, Fig. 3a) and a polytomy in Bayesian trees (Fig. 3b). Contrary to other cibicidids, sequences of *C. pachyderma*, *C. variabilis* and *Cibicidoides* sp. 5305 diverge mostly in the fragment sA-s6 (nine locations with one nucleotide difference, and one location with a short insertion in *C. pachyderma*). In the fragment s14-sB, the region 37/f is identical for all species and there is only one divergent nucleotide in 43/e, which is very surprising because these two regions can usually distinguish foraminiferal species (Pawlowski and Lecroq, 2010). Nevertheless, these results confirm the inconsistency between low genetic and high morphological variability already observed in the clade *C. wuellerstorfi* + *C. ungerianus* + *Cibicidoides* sp. (Schweizer et al., 2009). It also shows the limitation of the SSU rDNA to resolve species relationships in Cibicididae.

3.3.2. ITS rDNA

To help resolving the phylogenetic relationship of *C. variabilis* to other clade members, we sequenced the more variable ITS region for this species and its close relatives. We obtained 22 ITS sequences from *C. variabilis* with two specimens sequenced from each location: specimens 6484 (6 clones) and 6486 (1 clone) from Seno Ottway and specimens 6200 (8 clones) and 6205 (7 clones) from Punta Huinay. Despite many attempts, it was not possible to obtain ITS sequences from *Cibicidoides* sp. 5305 and *C. pachyderma* to compare them with the *C. variabilis* sequences. *C. lobatulus*, *C. wuellerstorfi* and *C. ungerianus* (sequences from Pawlowski et al., 2007). were used as an outgroup.

The analysis of ITS sequences within *C. variabilis* shows a certain genetic differentiation between examined specimens (Fig. 4). Sequences of isolates 6486, 6200 and 6205 are very similar to each other (< 1%), whereas the sequences of isolate 6484 are well separated from that group with a divergence of about 2.5% (Fig. 4). The ITS sequences show a high homogeneity of copies within a single specimen. This confirms that the ITS is useful for studying the intraspecific variations in benthic foraminifers, as previously shown by several studies (Grimm et al., 2007; Pawlowski et al., 2007; Schweizer et al., 2005; Tsuchiya et al., 2003, 2008, 2009).

4. Conclusions

Our results show clearly that *C. dispars* and *C. variabilis* are well separated from other shallow water cibicidids. This is particularly the case for *C. lobatulus,* with which they were sometimes put in synonymy. *Cibicidoides dispars* is well characterized genetically, falling in a clear separate clade and forming a sister group to *C. wuellerstorfi* and *Cibicidoides* sp. 5227 and sp. 2524. Unfortunately, *C. dispars* is difficult to recognize morphologically, with the major criterion of heterogeneous porosity not always present. On the other hand, *C. variabilis* is easily characterized morphologically by its chaotic growth in the adult stages, yet it is genetically very close to *C. pachyderma*. Further specimens are required to improve the morphological characterization of *C. dispars* and ITS sequences from *C. pachyderma* are needed to better resolve the clade *C. pachyderma* + *C. variabilis*, and to explore the intra-specific variation of *C. variabilis*.

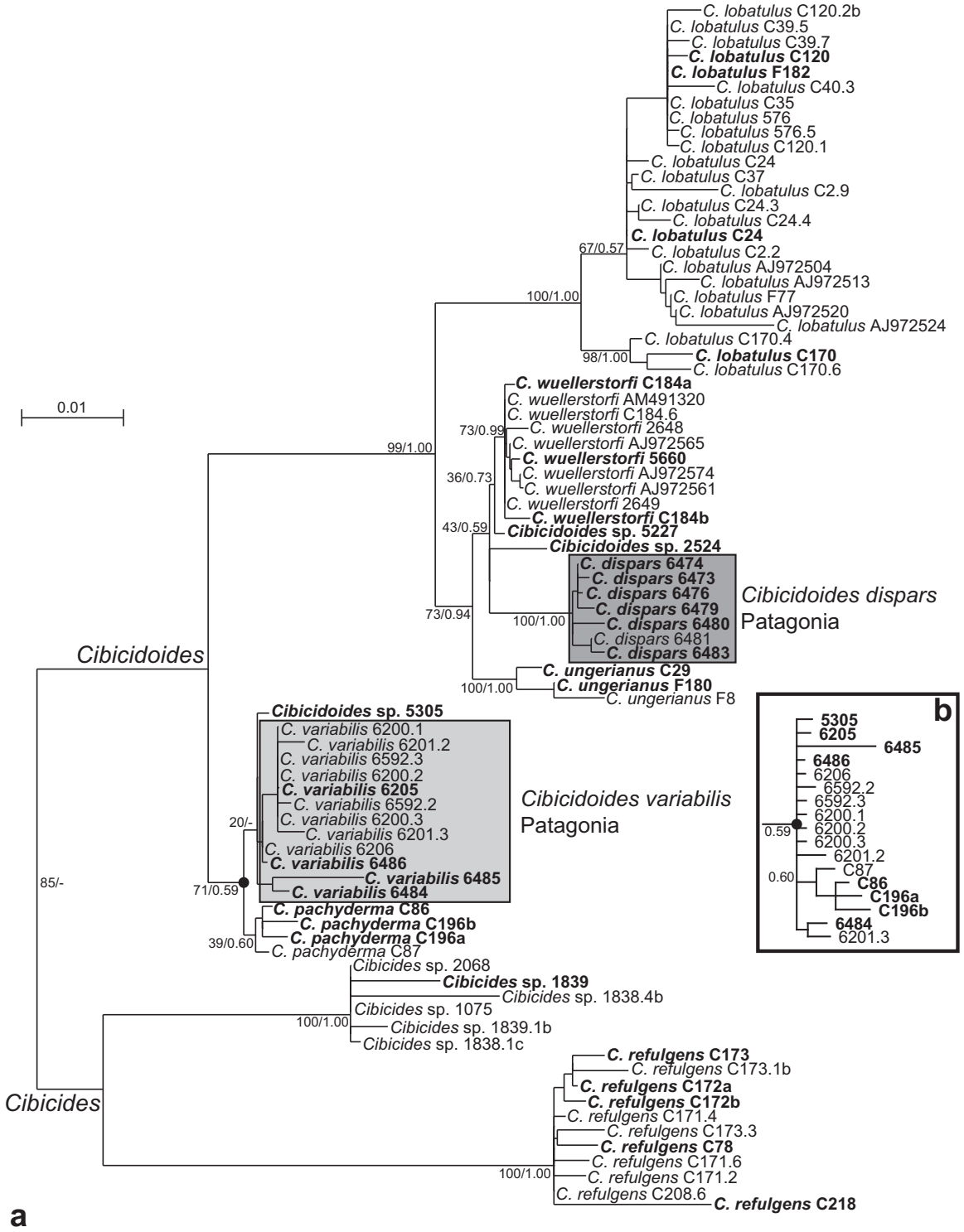

Fig. 3. **a**: molecular phylogeny of cibicidids with partial SSU rDNA sequences (concatenated fragments sA-s6 and s14-sB in bold) using the ML method (GTR + I + Γ). The tree is rooted on *Cibicides refulgens* and *Cibicides* sp.; BS values for ML and PP for BA are indicated at the nodes; **b**: detail of the Bayesian tree showing the clade of *C. pachyderma*, *Cibicidoides* sp. 5305 and *C. variabilis*. The black dot shows the same node in both analyses.

Considering the environments where the *Cibicidoides* investigated to date live, it is very interesting to observe that the neritic species examined here (*C. dispars* and *C. variabilis*) are closely related either to an abyssal (*C. wuellerstorfi*) or to a bathyal (*C. pachyderma*) species. This would suggest that the passage from shallow to deeper water, or vice versa, occurred at least twice in the genus *Cibicidoides*. Better resolution of relationships within this genus may provide new insight into the depth migration of benthic foraminifers and the origin of deep-sea species.

Moreover, there is a low genetic variability observed between species sampled in geographically distant areas: *C. pachyderma*

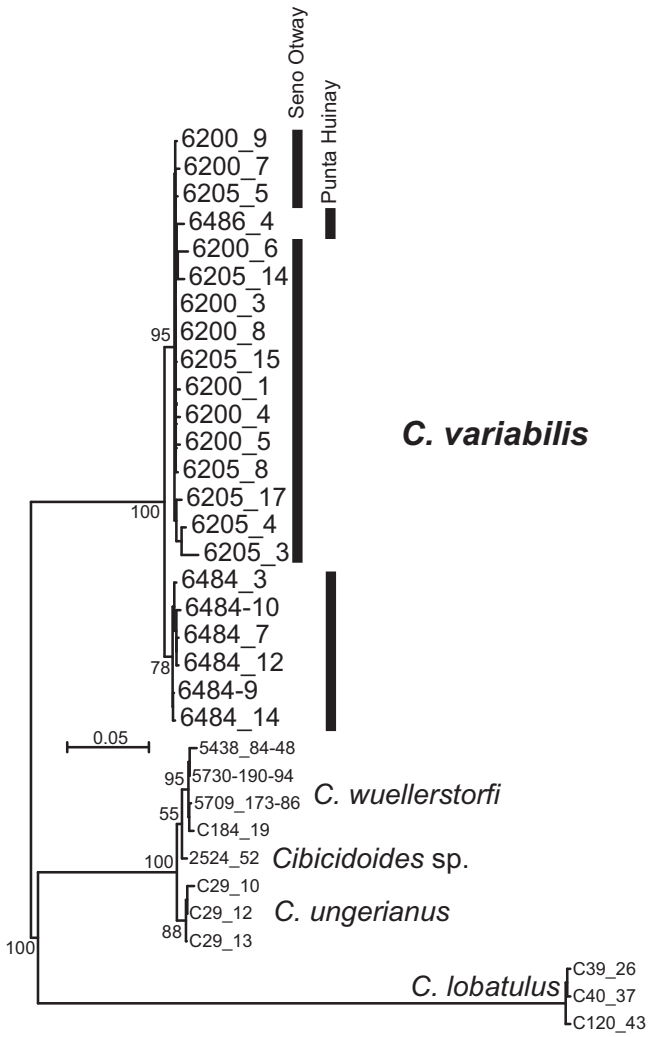

Fig. 4. BIONJ analysis of *C. variabilis* ITS rDNA sequences with the K2P model. The tree is rooted on *C. lobatulus*, *C. wuellerstorfi* and *C. ungerianus* and BS values are indicated at the nodes.

off Portugal, *C. variabilis* from Patagonia and *Cibicidoides* sp. from Svalbard. It is already clear that *C. pachyderma* and *C. variabilis* are well separated morphologically, but it would be interesting to study the population of *Cibicidoides* from Svalbard (no picture is available for *Cibicidoides* sp. 5305) to see if they can also be identified as *C. variabilis* morphologically.

Taxonomic list

Cibicidoides dispars (d'Orbigny, 1839)

1839. *Truncatulina dispars* – d'Orbigny, p. 38, pl. 5, figs. 25–27.

1932. *Truncatulina dispars* – Heron-Allen and Earland, p. 420, pl. 14, figs. 32–34.

1980. *Cibicides dispars* – Boltovskoy et al., p. 24, pl. 8, figs. 12–16.

1993. *Cibicides dispars* – Marchant, p. 64, pl. 1, fig. 5.

2002. *Cibicides dispars* – Hayward et al., pl. 1, figs. 22–24.

2003. *Cibicides dispars* – Hayward et al., fig. 4, R–T.

2007. *Cibicides dispars* – Hromic, fig. 3e.

2008. *Cibicides dispars* – Cusminsky and Whatley, pl. 2, fig. 4.

Cibicidoides variabilis (d'Orbigny, 1826)

1826. *Truncatulina variabilis* – d'Orbigny.

1932. *Truncatulina variabilis* – Heron-Allen and Earland, p. 420–421, pl. 14, figs. 36–38 (fig. 39 is not C. variabilis!).

1970. *Cibicides variabilis* – Boltovskoy and Theyer, p. 319, pl. 2, fig. 3.

1980. *Cibicides variabilis* – Boltovskoy et al., p. 25, pl. 9, figs. 12–17.

1993. *Cibicides variabilis* – Marchant, p. 64, pl. 1, fig. 6.

1995. *Cibicides variabilis* – Zapata et al., p. 53, figs. 8–9.

2000. *Cibicides variabilis* – Zapata and Olivares, p. 56.

2006. *Cibicidella variabilis* – Langer and Schmidt-Sinns, pl. 12, figs. 10–14.

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