Chapter 3

Molecular phylogeny of *Cibicides*, *Cibicidoides* and related genera (Rotaliida, Foraminifers): taxonomic implications

Abstract

Cibicidids comprise several species of rotaliid foraminifera that are widely used as proxies of marine paleoenvironments. On the basis of test form and structure several genera of cibicidids have been erected, of which some have been placed in different families and superfamilies. To test the monophyly of the cibicidids and to infer their phylogenetic relationships, we obtained partial small-subunit ribosomal DNA (SSU rDNA) sequences of six common species: *kullenbergi, lobatulus, pachyderma, refulgens, ungerianus* and *wuellerstorfi.* Phylogenetic analyses of our sequence data show that the cibicidids group together, albeit their monophyly is not strongly supported. Among the six species, two (*lobatulus* and *wuellerstorfi*) form well defined clades, branching together in all analyses. Two species (*kullenbergi*, *pachyderma*) form a single clade, while one (*refulgens*) splits into two clades, possibly indicating the existence of two cryptic species. The sixth species, *ungerianus*, represented by a single sequence, branches as a sister group to *wuellerstorfi.* The wide morphological variations observed in *lobatulus* seem to be due mainly to environmental factors, since regularly and irregularly shaped specimens (ecophenotypes) group together in the molecular analyses. In view of our analyses, the distinction between planoconvex *Cibicides* and biconvex *Cibicidoides* and the placement of cibicidids in different superfamilies is not justified. Our data suggest that all species examined here could be classified in one unique family, and, for the time being, in a single genus, *Cibicides* de Montfort, 1808. This genus has been defined by a low trochospiral coil with an evolute spiral side and an involute umbilical side, and a simple slit as an aperture, located near the peripheral margin and edged by a lip.

Keywords: Benthic foraminifera; Rotaliida; Cibicidids; *Cibicides; Cibicidoides*, SSU rDNA; Molecular phylogeny

3.1. Introduction

Cibicidids play an important role in the fossil record as proxies of marine paleoenvironmental conditions like trophic state (e.g. Altenbach & Sarnthein, 1989), oxygen (Kaiho, 1994), and paleodepth (e.g. Wright, 1978; Van der Zwaan et al., 1999; Van Hinsbergen et al., 2005). Furthermore, cibicidids are frequently used in stable carbon and oxygen isotopic analyses. Most species have an epibenthic or shallow infaunal microhabitat (Murray, 2003). *Cibicides wuellerstorfi* (Schwager, 1866), which is the most commonly used species in stable isotope studies (Murray, 1991), is considered to reliably reflect bottom water oxygen and carbon isotope ratios because it is an epibenthic species (Lutze & Thiel, 1989; Schmiedl et al., 2004; but see Mackensen et al., 1993). To construct proper down-core isotope curves, it is important to use one single species instead of a mix of different species (Murray, 1991; Schmiedl et al., 2004). Therefore, the status and recognition of the different species play an important role, not only for evolutionary purposes, but also in paleoecology.

The present classification of the cibicidids is entirely based on morphological characteristics and there is some confusion about the generic status of the different species. The species examined in this paper have been and still are classified in various genera (the most commonly used names are shown between brackets, see taxonomic notes in the appendix for more details): *Anomalina* d'Orbigny, 1826, *Cibicides* de Montfort, 1808 (*Cibicides refulgens* de Montfort, 1808*, C. kullenbergi* Parker, 1953*, C. lobatulus* (Walker and Jacob, 1798), *C. pachyderma* (Rzehak, 1886), *C. ungerianus* (d'Orbigny, 1846), *C. wuellerstorfi*), *Cibicidoides* Thalmann, 1939 (*Cibicidoides kullenbergi, C. pachyderma*), *Fontbotia* Gonzalez-Donoso & Linares, 1970 (*Fontbotia wuellerstorfi*), *Heterolepa* Franzenau, 1884 (*Heterolepa kullenbergi*), *Lobatula* Fleming, 1828 (*Lobatula lobatula*), *Planulina* d'Orbigny, 1826 (*Planulina wuellerstorfi*), *Truncatulina* d'Orbigny, 1826.

Among the validated generic names, *Cibicides* was the most commonly used for this group of species during the first half of the 20th century. *Cibicidoides* was initially described as a subgenus of *Cibicides* in 1936 by Brotzen and validated by Thalmann (1939) upon the designation of a subgenotype. However, *Cibicidoides* only became a widely used genus name for biconvex forms since the end of the 1970s. *Lobatula*, *Truncatulina* and *Heterolepa* were considered junior synonyms of *Cibicides* by Galloway & Wissler (1927) and Cushman (1928). *Planulina* and *Fontbotia* have been used as generic names for *wuellerstorfi* (e.g. Van Morkhoven et al., 1986; Holbourn

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& Henderson, 2002; respectively Gonzalez-Donoso & Linares, 1970; Loeblich & Tappan, 1988). However, *Planulina* differs from *Cibicides* by the partially evolute umbilical side and from *Cibicidoides* by the planoconvex shape of the test, while *Fontbotia* was regarded as a junior synonym of *Cibicides* (Sen Gupta, 1989), *Cibicidoides* (Whittaker, 1988) or *Planulina* (Revets, 1996).
Many authors have considered the cibicidids as a monophyletic group and have placed them together within the family Anomalinidae Cushman, 1927 (Cushman, 1928; Reiss, 1958), Rotaliidae Reuss, 1860 (Galloway, 1933), or Cibicididae Cushman, 1927 (Hofker, 1956). Loeblich & Tappan (1964) introduced a classification in which they placed *Cibicides* and *Cibicidoides* in two different superfamilies, distinguished by the crystallographic structure of the wall: the Orbitoidacea (radial) for *Cibicides* and the Cassidulinacea (granular) for *Cibicidoides,* together with *Heterolepa.* The placement of *Cibicidoides* and *Heterolepa* in a separate superfamily was based on the granular structure of the wall compared to the radial wall of the other cibicidids (Loeblich & Tappan, 1962). In their later classification (1988), Loeblich & Tappan maintained a division of the cibicidids over different superfamilies. The wall structure is considered of great importance in the classifications of Loeblich & Tappan (1964, 1988). Towe & Cifelli (1967), however, showed that this difference, which seems huge when observed in polarized light, is a matter of orientation of the crystals: the same crystal morphology can produce different optical orientations, and conversely, similar optical characteristics can be generated by different crystal forms. These authors (1967, p. 754) demonstrated that *C. refulgens*, which was first considered having a granular wall, and later a radial one, has in fact optical attributes of both radial and granular wall structures. They concluded that the dichotomy radial versus granular cannot be used as a major criterion for higher taxonomic levels (Towe & Cifelli, 1967, p. 755).
Summarizing, there are two concepts of the classification of cibicidids in the more recent works: they are either united in a single family (Haynes, 1981, Sen Gupta, 2002) or separated in different superfamilies (Loeblich & Tappan, 1988, 1992; Revets, 1996).
Here, we use SSU rDNA sequences to investigate the phylogeny of six Recent species of cibicidids and to establish their relationships with other rotaliids. Until now, only three sequences of cibicidids have been deposited in the EMBL/GenBank data base: *C. refulgens* (AJ514839) (Pawlowski et al., 2003), *C. wuellerstorfi* (AY934741) and *C. lobatulus* (AY934742) (Schweizer et al., 2005). These sequences correspond to the 3' end fragment of the SSU rDNA, which is widely used in foraminiferal phylogeny (e.g. Pawlowski, 2000; Holzmann et al., 2003; Darling et al., 2004; Ertan et al., 2004). We extended this dataset by the addition of 53 new sequences of the 3' end fragment and 37 new sequences of a fragment situated at the 5' beginning of the SSU. Phylogenetic analyses of these combined sequence data indicate that the cibicidids form a

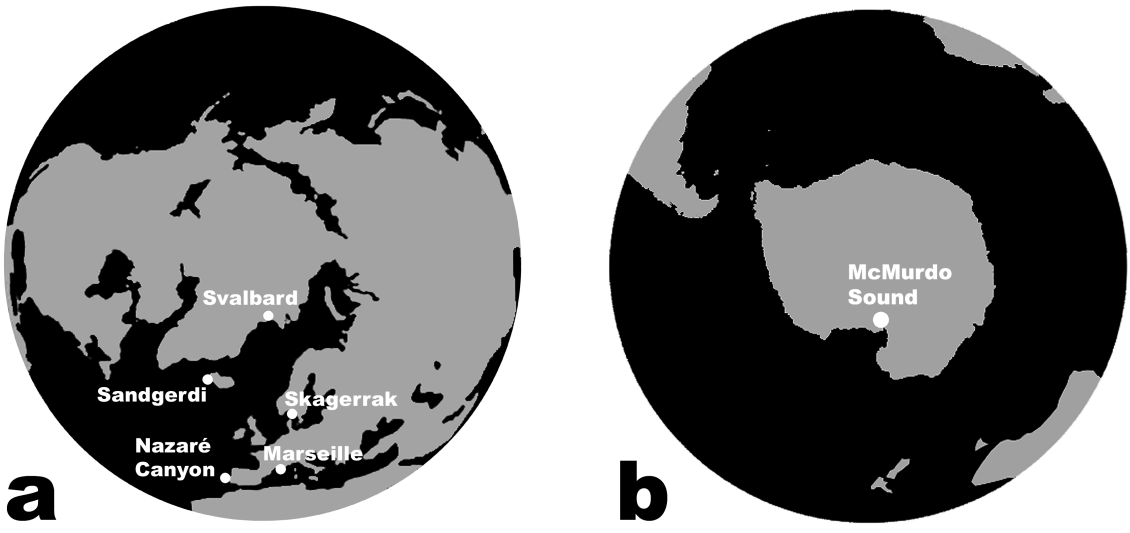

Figure 3.1. Maps showing the sampling sites of the northern (a) and southern (b) hemispheres.

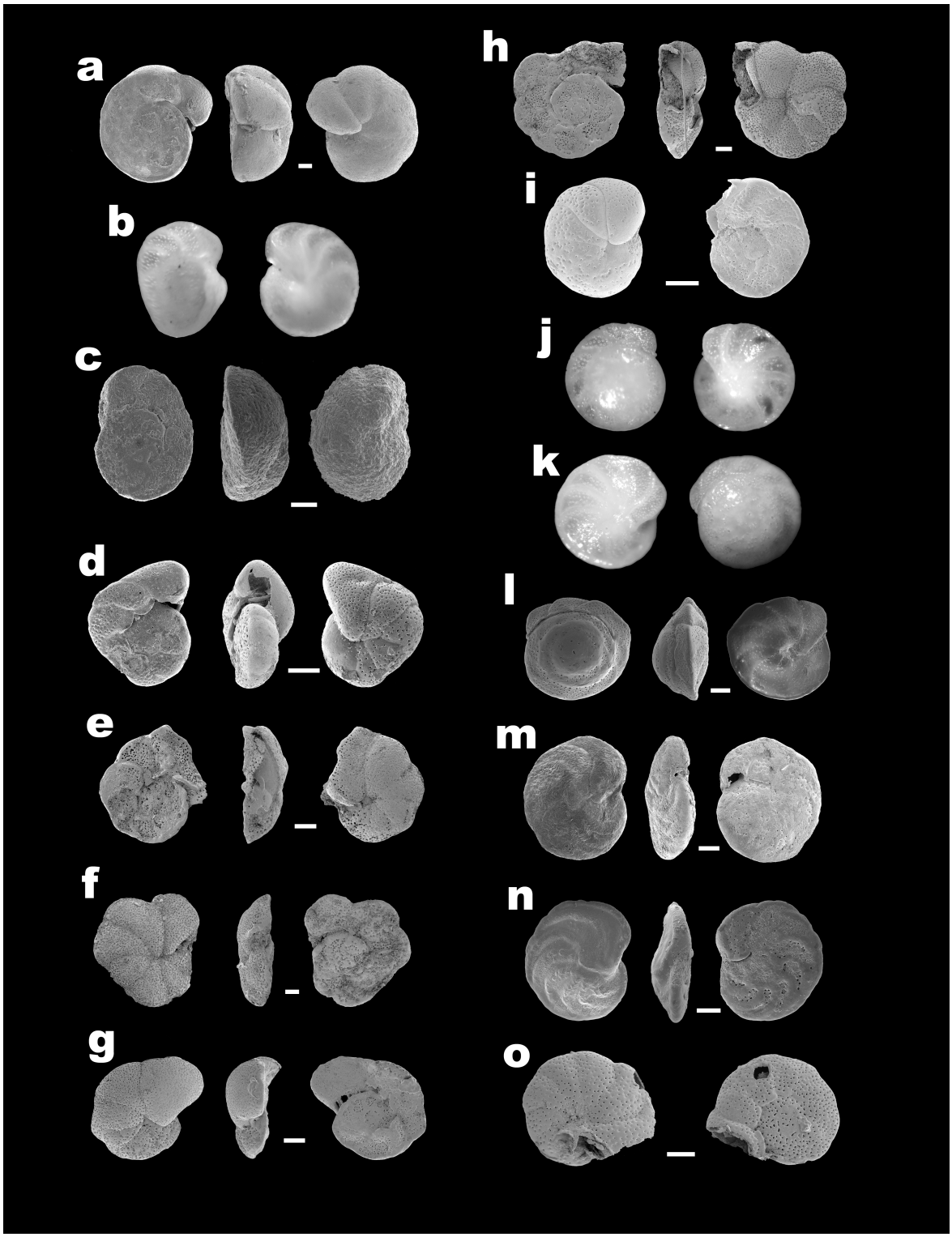

Figure 3.2. SEM pictures and light photomicrographs (b, j, k) of the studied specimens of *Cibicides* (u: umbilical side, s: spiral side, p: profile). Except (a), (c) and (m), all the pictures correspond to DNA samples (DNA number is indicated in brackets after the species name). (a) *C. refulgens* from Antarctica (u, p, s), (b) *C. refulgens* (C78) from the Mediterranean (s, u), (c) *C. refulgens* from the Mediterranean (s, p, u), (d) *C. lobatulus* (C2) from Iceland (s, p, u), (e) *C. lobatulus (*C35) from Oslo Fjord (s, p, u), (f) *C. lobatulus* (C37) from Oslo Fjord (u, p, s), (g) *C. lobatulus* (C39) from Oslo Fjord (u, p, s), (h) *C. lobatulus* (C40) from Oslo Fjord (s, p, u), (i) *C. lobatulus* (C120) from Skagerrak (u, s), (j) *C. kullenbergi* (C86) from Portugal (s, u), (k) *C. kullenbergi* (C87) from Portugal (u, s), (l) *C. pachyderma* (C196) from Portugal (u, p, s), (m) *C. wuellerstorfi* from Svalbard (u, p, s), (n) *C. wuellerstorfi* (C184) from Portugal (u, p, s), (o) *C. ungerianus* (C29) from Oslo Fjord (u, s). Scale= 100 μm

monophyletic group, which branches closely to *Melonis* de Montfort, 1808 and *Pullenia* Parker and Jones, 1862. The relationships within this group and molecular versus morphological variations in some species are discussed in this paper.

3.2. Material and Methods

3.2.1. Sample collection

Living individuals of cibicidids were obtained from the North Atlantic, the North Sea, the Mediterranean and the Southern Ocean (Fig. 3.1). Shallow water samples were collected by SCUBA diving or from intertidal rocks; they were kept at a temperature close to the one observed where they were collected. Deeper-water samples were obtained by boxcoring or multicoring. The top few centimeters of sediment were collected, immediately sieved and kept in the refrigerator at 4°C. Live specimens, identified by their natural coloration (mainly pinkish) were cleaned, picked and dried on Chapman slides (see Schweizer et al., 2005 for details). Most of the specimens were subsequently pictured with scanning electron microscope (SEM) or a camera connected to a dissection microscope, before DNA extraction (Fig. 3.2).

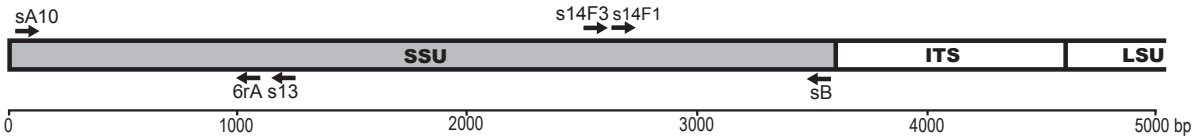

Figure 3.3. Schematic representation of the rRNA genes and the approximate position of the primers used in this study.

3.2.2. DNA extraction, PCR amplification, cloning and sequencing

DNA was extracted from single specimens using DOC lysis buffer (Pawlowski, 2000) and from samples containing multiple specimens by DNeasy Plant Mini Kit (Qiagen).
Two fragments of the SSU, each about 1,000 nucleotides in length, were examined (Fig. 3.3). The first fragment starting at the 5' end of the SSU was amplified with the primers sA10 and s13 and reamplified using primers sA10 and s6rA. The second fragment placed at the 3' end of the SSU was amplified using the primer pair s14F3 and sB and reamplified with the primer pair s14F1 and sB. The sequences of all these primers are available in Table 2.3. Both fragments were amplified by PCR (polymerase chain reaction) in a total volume of 50µl. The thermal cycle parameters consisted of 40 cycles of 30s at 94°C, 30s at 50°C and 120s at 72°C, followed by 5min at 72°C for final extension. Reamplification was carried out using 35 cycles of 30s 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). PCR products obtained from the 3' end fragment of DNA samples 1075, 1994, 2524, 2648, 2649, 3623, C29, C35, C37, C78, C86, C87, U27 (see Table 3.1) were sequenced directly. All other PCR products were ligated in the pGEM-T Vector (Promega) or the Topo Cloning vector (Invitro Gene), and cloned using ultracompetent cells XL2-Blue MRF' (Stratagene). Sequencing reactions were prepared using an ABI-PRISM Big Dye Terminator Cycle Sequencing Kit and analysed with DNA sequencers ABI-377 or ABI-PRISM 3100 (Applied Biosystems), all according to the manufacturer's instructions.
New sequences have been deposited in the EMBL/GenBank database; their accession numbers are indicated in Table 3.1.

Table 3.1. List of new SSU sequences and origin of DNA samples. Asterisks indicate sequences previously published.

Access # A10	14F1	Species	DNA #	Collection site	Cells
DQ205389	AY934747*	*Bulimina marginata*	3599	Oslo Fjord, Norway	130
DQ205355	AY934737*	*Cassidulinoides porrectus*	3924	Terranova Bay, Antarctica	3
DQ205369	DQ195545, DQ195583, DQ195584	*Cibicides lobatulus*	C170	Marseille, France	1
	DQ195576, DQ195585	*Cibicides lobatulus*	C2	Sandgerdi, Iceland	1
DQ205371	DQ195547, DQ195577, DQ195578, DQ195579	*Cibicides lobatulus*	C24	Oslo Fjord, Norway	1
	DQ195580	*Cibicides lobatulus*	C35	Oslo Fjord, Norway	1
	DQ195581	*Cibicides lobatulus*	C37	Oslo Fjord, Norway	1
	AY934742*, DQ195586	*Cibicides lobatulus*	C39	Oslo Fjord, Norway	1
	DQ195587	*Cibicides lobatulus*	C40	Oslo Fjord, Norway	1
DQ205372	DQ195548, DQ195561, DQ195562	*Cibicides lobatulus*	C120	Skagerrak, Sweden	1
	DQ195573, DQ195574	*Cibicides lobatulus*	576	Skagerrak, Sweden	5
DQ205377, DQ205378	DQ195552, DQ195553, DQ195563	*Cibicides pachyderma*	C196	Nazaré Canyon, Portugal	1
DQ205376	DQ195551	*Cibicides kullenbergi*	C86	Nazaré Canyon, Portugal	1
	DQ195575	*Cibicides kullenbergi*	C87	Nazaré Canyon, Portugal	1
	DQ195564	*Cibicides refulgens*	1075	McMurdo Sound, Antarctica	1
	DQ195566, DQ195567	*Cibicides refulgens*	1838	McMurdo Sound, Antarctica	1
DQ205368	DQ195544, DQ195565	*Cibicides refulgens*	1839	McMurdo Sound, Antarctica	1
	AJ514839*	*Cibicides refulgens*	2068	McMurdo Sound, Antarctica	10
DQ205367	DQ195543	*Cibicides refulgens*	C78	Gulf of Lions, France	1
	DQ195568, DQ195569, DQ195570	*Cibicides refulgens*	C171	Marseille, France	1
DQ205365, DQ205366	DQ195541, DQ195542	*Cibicides refulgens*	C172	Marseille, France	1
DQ205364	DQ195540, DQ195571, DQ195572	*Cibicides refulgens*	C173	Marseille, France	1
	DQ195582	*Cibicides refulgens*	C208	Marseille, France	1

A10	14F1	Species	DNA #	Collection site	Cells
DQ205375	DQ195550	*Cibicides* sp.	2524	North Atlantic	1
DQ205370	DQ195546	*Cibicides ungerianus*	C29	Oslo Fjord, Norway	1
	DQ195560	*Cibicides wuellerstorfi*	2648	Svalbard, Norway	
	DQ195559	*Cibicides wuellerstorfi*	2649	Svalbard, Norway	
DQ205373, DQ205374	AY934741*, DQ195549, DQ195558	*Cibicides wuellerstorfi*	C184	Setubal Canyon, Portugal	1
DQ205360	DQ195538	*Discorbis rosea*	753	Florida, USA	1
DQ205386	DQ195557	*Epistominella exigua*	3623	Weddell Sea, Antarctica	1
DQ205384, DQ205385	AY934750*, DQ195556	*Epistominella vitrea*	2060	Cape Evans, Antarctica	4
DQ205362	DQ195539	*Hyalinea balthica*	3604	Oslo Fjord, Norway	
DQ205354	AJ504685*	*Islandiella* sp.	2643	Svalbard, Norway	
DQ205379	AY934753*	*Melonis pompilioides*	1400	Skagerrak, Sweden	1
DQ205361	AJ504684*	*Planorbulina mediterranensis*	142	Golfe du Morbihan, France	1
DQ205382, DQ205383	AY934755*, DQ195555	*Pullenia subcarinata*	1148	McMurdo Sound, Antarctica	1
DQ205380, DQ205381	AY934754*, DQ195554	*Pullenia subcarinata*	1850	McMurdo Sound, Antarctica	1
DQ205357	AY914563*	*Rectuvigerina phlegeri*	U239	Nazaré Canyon, Portugal	
DQ205363	DQ195588	Unknown rotaliid	3675	Culture	100
DQ205387	AY934744*	*Stainforthia fusiformis*	3965	Skagerrak, Sweden	150
DQ205390	AY914568*	*Trifarina earlandi*	1994	McMurdo Sound, Antarctica	10
DQ205356	AY914565*	*Trifarina earlandi*	2187	McMurdo Sound, Antarctica	5
DQ408637	DQ408637	*Trochammina hadai*	95	Hamana Lake, Japan	1
DQ205359	DQ195537	*Uvigerina peregrina*	U27	Oslo Fjord, Norway	9
DQ205358	AY914571*	*Uvigerina peregrina*	U32	Oslo Fjord, Norway	2

3.2.3. Phylogenetic analysis

Sequences were aligned manually using Seaview (Galtier et al., 1996). Three sequence datasets were analysed. The first dataset includes a total of 2632 aligned sites from concatenated 3' and 5' fragments for 15 sequences of cibicidids, 27 sequences of other rotaliids and three sequences of textulariids, taken as an outgroup. The second dataset comprises 2357 aligned sites from the concatenated fragments for the 15 sequences of cibicidids, the 10 most closely related sequences of rotaliids and the Nummulitidae and *Pararotalia* as the outgroup. The third dataset includes 1013 aligned sites from the 3' fragment with 47 sequences of cibicidids and four sequences of *Pullenia subcarinata* used as an outgroup.

The maximum likelihood (ML) trees were obtained using PhyML 2.4.4 (Guindon & Gascuel, 2003). To assess the reliability of internal branches, the bootstrap support (BS) values were calculated by PhyML, with 100 replicates. Bayesian analyses were done with MrBayes 3.1.1 (Huelsenbeck & Ronquist, 2001). Two independent analyses were performed at the same time with four simultaneous chains run for 1,000,000 generations, and sampled every 100 generations with 1,000 initial trees discarded as burn-in. The posterior probabilities (PP), calculated during the Bayesian analysis, estimated the reliability of internal branches. Both ML and Bayesian analyses were performed using the GTR+I+G model as suggested by Modeltest 3.7 (Posada & Crandall,

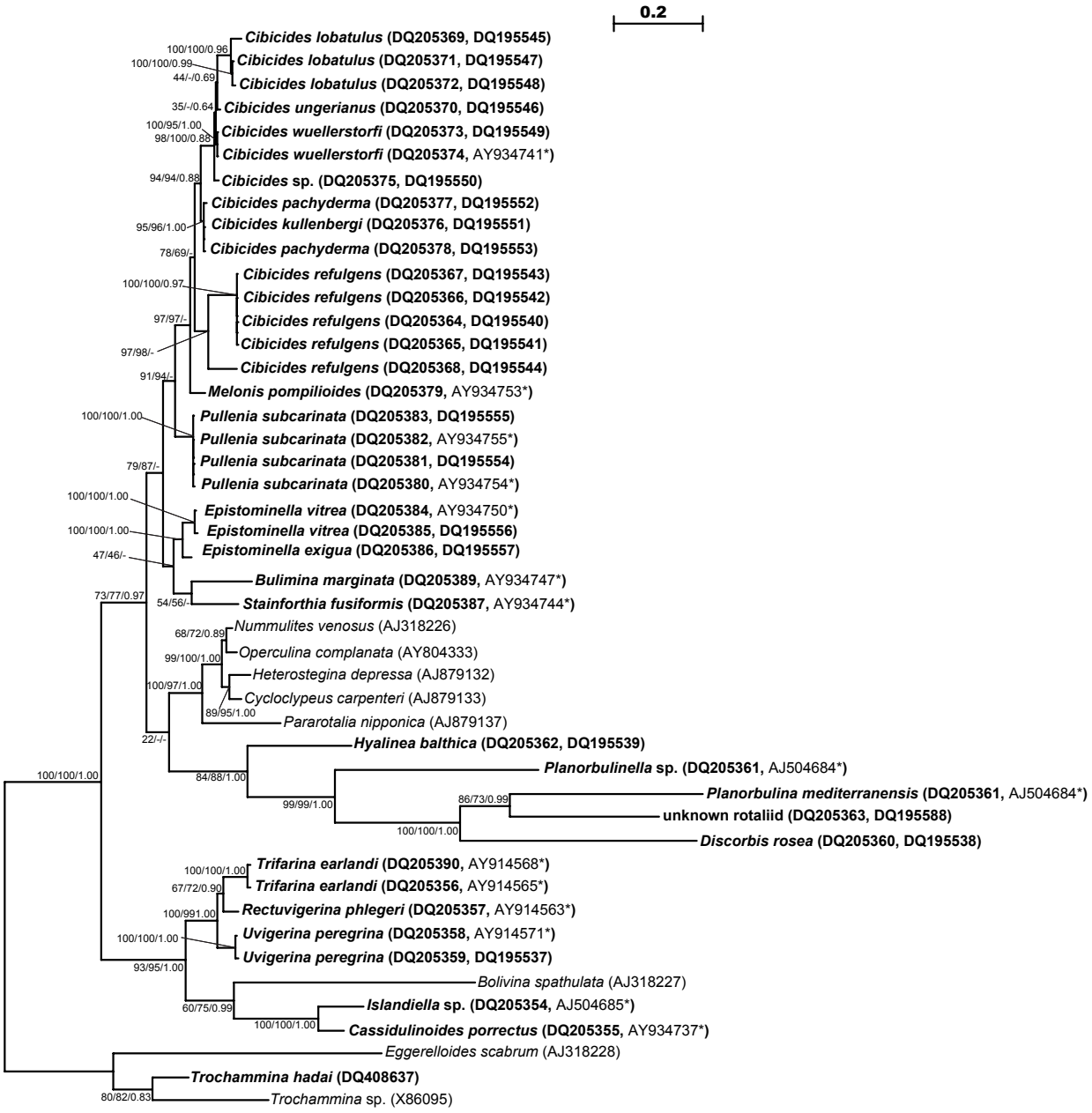

Figure 3.4. Phylogeny of Rotaliida inferred from partial SSU rDNA sequences (5' and 3' end fragments) using the ML (HKY+I+G) method (2632 aligned sites). Values are given for internal nodes for HKY, GTR and PP. Species names written in bold designate new sequences, the others were taken from GenBank (accession numbers in brackets).

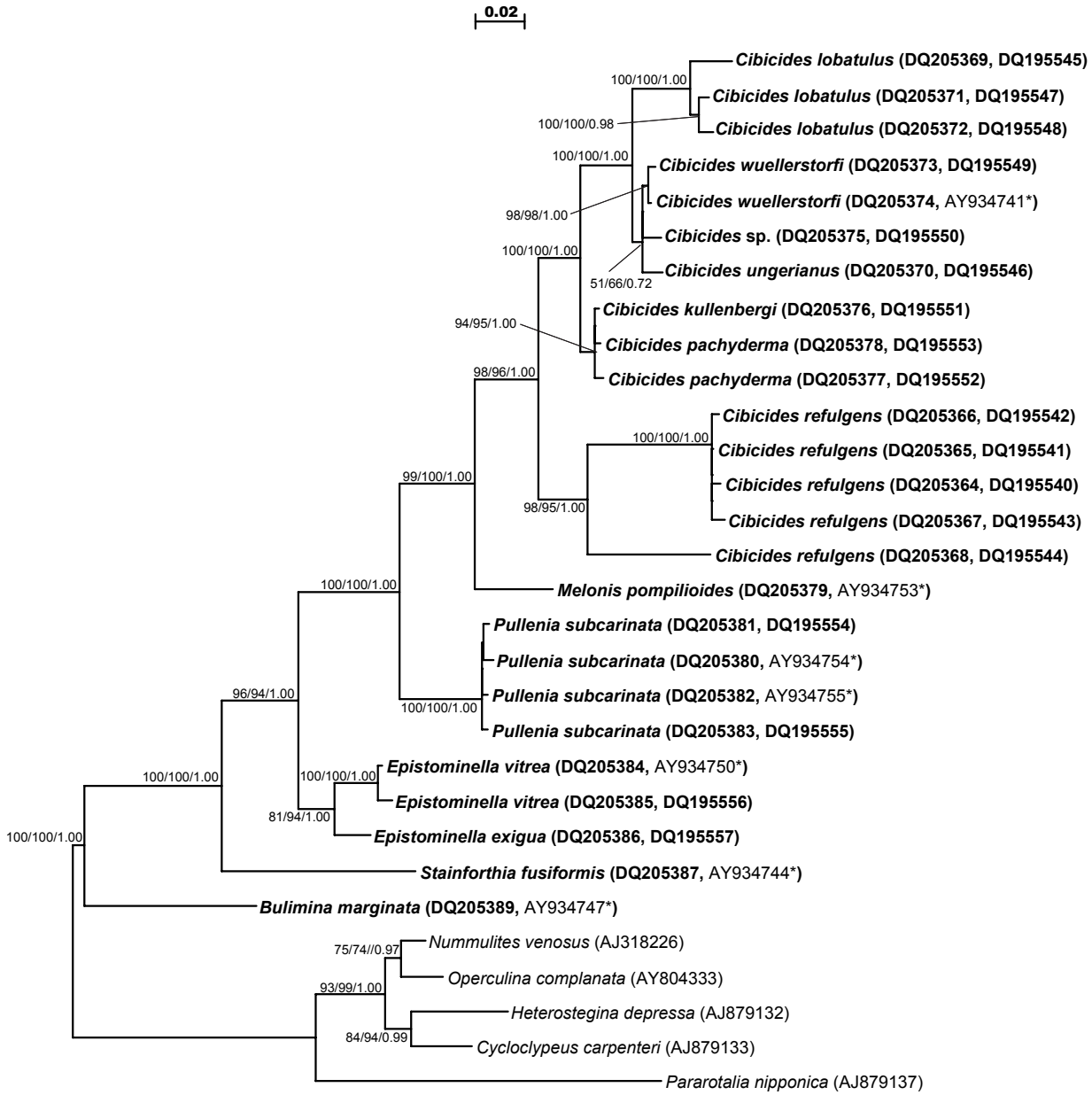

Figure 3.5. Phylogeny of *Cibicides* and closely related species inferred from partial SSU rDNA sequences (5' and 3' end fragments) using the ML (HKY+I+G) method (2357 aligned sites). Values are given for internal nodes for HKY, GTR and PP. Species names written in bold designate new sequences, the others were taken from GenBank (accession numbers in brackets).

1998). The GTR or General Time Reversible model allows the transition and transversion rates to be different (Lanave et al., 1984; Rodriguez et al., 1990). To correct for among-site rate variations, the proportion of invariable sites (I) and the α parameter of γ distribution (G), with eight rate categories, were estimated by the programs and taken into account in all analyses. Additionally, the HKY model (Hasegawa et al., 1985), allowing transitions and transversions to have potentially different rates, was applied with PhyML.

3.3. Results

First, we analysed the two concatenated fragments with all the sequences available to test the monophyly of cibicidids and to infer their position among Rotaliida Delage and Hérouard, 1896. As shown in Fig. 3.4, the 15 sequences of cibicidids group together in the ML tree, albeit the bootstrap support for this grouping is rather weak (78% BS with HKY/69% BS with GTR). This support increases up to 97% BS (HKY) if the sequence of *Melonis pompilioides* (Fichtel and Moll, 1798), which branches as a sister group to cibicidids is removed (data not shown).

The cibicidids group together with *M. pompilioides*, *Pullenia subcarinata* (d'Orbigny, 1839), *Epistominella* Husezima and Maruhasi, 1944 (*E. exigua* (Brady, 1884) and *E. vitrea* Parker, 1953), *Stainforthia fusiformis* (Williamson, 1858) and *Bulimina marginata* d'Orbigny, 1826 in a reasonably supported clade (79% BS with HKY/87% BS with GTR). Three other major groupings in the ML tree are the sub-clade of Nummulitidae de Blainville, 1827 + *Pararotalia nipponica* (Asano, 1936) (100% BS with HKY/97% BS with GTR) and the sub-clade of *Hyalinea balthica* (Schroeter, 1783) + *Planorbulinella* sp. + *Planorbulina mediterranensis* d'Orbigny, 1826 + *Discorbis rosea* (d'Orbigny, 1826) + unkown rotaliid (84% BS with HKY/88% BS with GTR) grouped together and the clade of Uvigerinidae Haeckel, 1894 + Cassidulinidae d'Orbigny, 1839 + *Bolivina spathulata* (Williamson, 1858) (93% BS with HKY/95% BS with GTR*).* In the HKY analysis, the Nummulitidae + *Pararotalia* group with the *Hyalinea* + *Planorbulinella* + *Planorbulina* + *Discorbis* + unkown rotaliid clade, whereas in the GTR analysis, they group with the *Cibicides* + *Melonis* + *Pullenia* + *Epistominella* + *Stainforthia* + *Bulimina* clade. Two groups (uvigerinids – cassidulinids - *Bolivina* and *Hyalinea – Planorbulinella – Planorbulina* - unknown rotaliid - *Discorbis*) are also recognized in Bayesian analyses, with statistical support of 1.00 PP and a structure similar to the one found in the ML analysis. In the Bayesian tree the group *Epistominella + S. fusiformis + B. marginata + P. subcarinata + M. pompilioides + Cibicides* appears as paraphyletic, with the clade Nummulitidae + *P. nipponica* branching within it. With the exception of *C. refulgens*, the cibicidids form a monophyletic clade with 0.88 PP (data not shown).

To investigate the relationships between cibicidid species, we analysed the concatenated data for the clade *Cibicides + M. pompilioides + P. subcarinata + Epistominella + S. fusiformis + B. marginata*, using Nummulitidae and *P. nipponica* as an outgroup (Fig. 3.5). The resulting tree has almost the same topology as the one in Fig. 3.4, but the bootstrap values have substantially increased in almost every case. The topology of ML and Bayesian trees is similar. The clade of cibicidids is supported by 98% BS (HKY), 96% BS (GTR) and 1.00 PP. It branches as sister group to *M. pompilioides*, with 99% BS (HKY), 100% BS (GTR) and 1.00 PP. *Pullenia subcarinata* and *Epistominella* form successive sister groups with strong BS and PP values. Within the cibicidids, three well supported clades can be distinguished: the most basal *C. refulgens* clade (98% BS (HKY), 95% BS (GTR), 1.00 PP), the *C. pachyderma + C. kullenbergi* clade (94% BS (HKY), 95% BS (GTR), 1.00 PP), and the *C. ungerianus + Cibicides* sp. *+ C. wuellerstorfi + C. lobatulus* clade (100% BS (HKY and GTR), 1.00 PP).

The third dataset, including 47 cibicidid sequences, was analysed to examine intraspecific variations (Fig. 3.6), using the fragment 14F1-B. *Pullenia subcarinata* was chosen as an outgroup, because there were several sequences available for this species. All morphospecies form well supported groups, except *C. refulgens,* which splits into two clades, one grouping the specimens from Antarctica and branching as sister to all other cibicidids and the second comprising the specimens from the Mediterranean. The statistical support is good for most of the clades, although

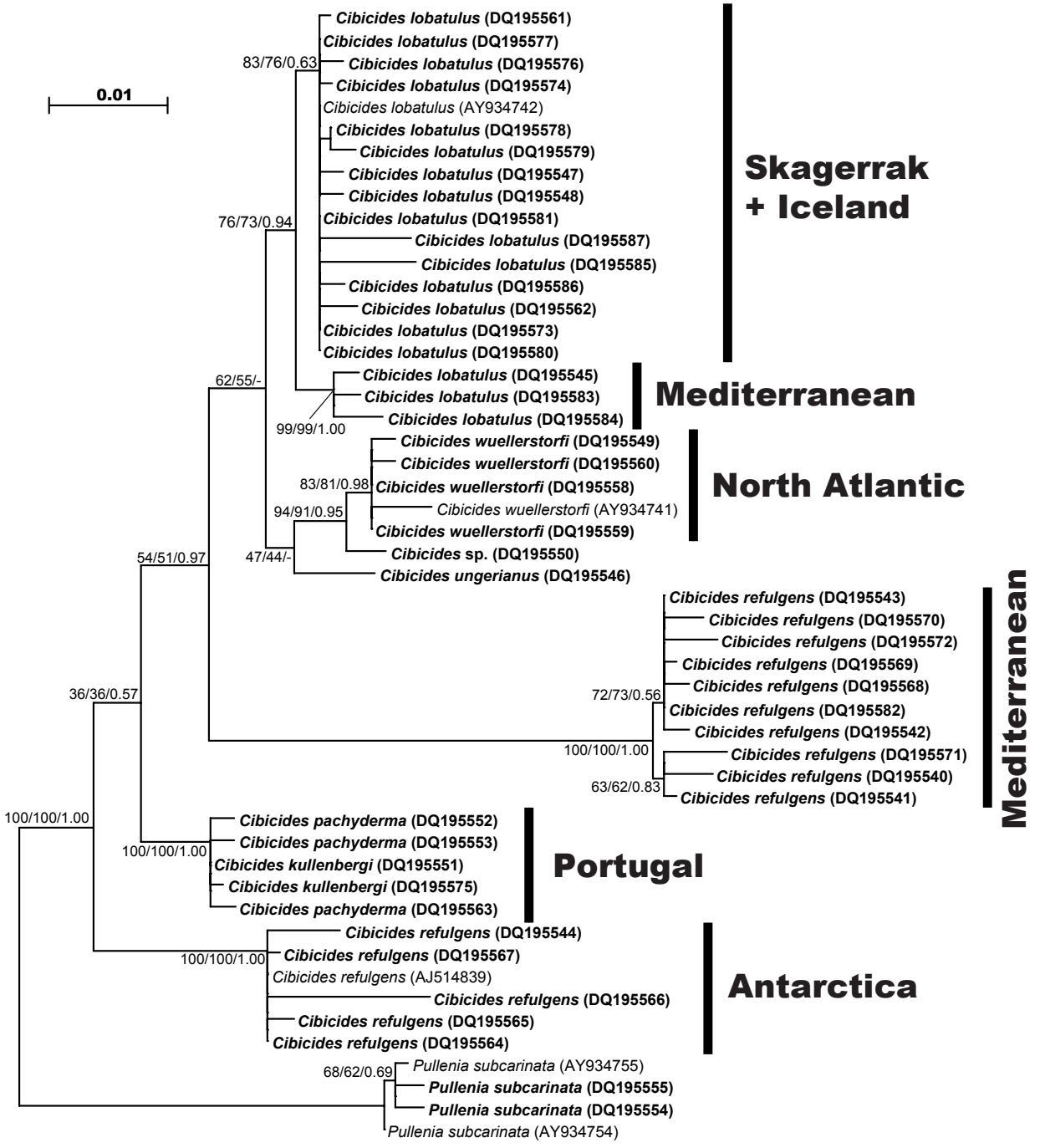

Figure 3.6. Phylogeny of *Cibicides* inferred from partial SSU rDNA sequences (3' end fragment) using the ML (HKY+I+G) method (1013 aligned sites). Values are given for internal nodes for HKY, GTR and PP. Species names written in bold designate new sequences, the other ones were taken from GenBank (accession numbers in brackets).

the nodes connecting the clades are not very well supported (below 75% BS in all cases). The Bayesian analysis confirms the ML topology except for the Antarctic population of *C. refulgens*, which branches as a sister group to *C. wuellerstorfi* (data not shown). *Cibicides lobatulus* forms a well defined group with clear geographical subgroups for populations from the North Atlantic and the Mediterranean. *Cibicides pachyderma* and *C. kullenbergi* branch together. The last taxa, *C. ungerianus* and *Cibicides* sp., represented by only one sequence each, group with *C. wuellerstorfi*.

3.4. Discussion

3.4.1. Are cibicidids monophyletic?

Phylogenetic analyses of our data showed that the cibicidids are monophyletic in ML trees but failed to support their monophyly in Bayesian trees. Comparing the results of analyses with different numbers of sites, we noticed that whether the clade of *Cibicides* shows up as monophyletic depends on the length of the examined sequences. When we analysed a shorter fragment of the SSU, which is traditionally used in foraminiferal phylogeny (e.g. Pawlowski, 2000; Holzmann et al., 2003; Darling et al., 2004; Ertan et al., 2004), the cibicidids neither grouped together in ML nor in Bayesian analyses (Fig. 2.7). By combining two fragments of the SSU we obtained more informative sites, and were able to establish the relationships among rotaliids more accurately. The analyses of combined fragments confirmed the phylogenetic position of the major groups and significantly increased the bootstrap support for most of the clades defined in a previous study (Schweizer et al., 2005, Fig. 7). Additional analyses show that these supports are even higher when complete SSU sequences are analysed (see Chapter 2).

Although the support for monophyly of cibicidids is not very strong, there is even less evidence to consider them as belonging to different superfamilies, as suggested by some morphology-based classifications (Loeblich & Tappan, 1964, 1988; Revets, 1996). Cibicidids share many morphological traits: the coarsely perforate wall made of hyaline lamellar calcite, the trochospiral coil with an evolute spiral side and an involute umbilical side, and the aperture, which is a simple slit edged by a lip and located near the peripheral margin on the umbilical side. Although they were split into different superfamilies on the basis of the optical properties of their wall microstructure by Loeblich & Tappan (1964, 1988), this criterion was already dismissed as inappropriate for classification of higher taxa (Towe & Cifelli, 1967, Deutsch Conger et al., 1977). Our molecular results have confirmed that and agree with the classifications which place all the cibicidids in a single family (Cushman, 1928, Galloway, 1933, Hofker, 1956, Reiss, 1958, Haynes, 1981, Sen Gupta, 2002).

The close relationship of cibicidids with *Melonis* and *Pullenia* may appear surprising in view of traditional taxonomy. *Melonis* and *Pullenia* belong to the superfamily Nonionacae Schultze, 1854 (Loeblich & Tappan, 1988). However, there are some morphological similarities between

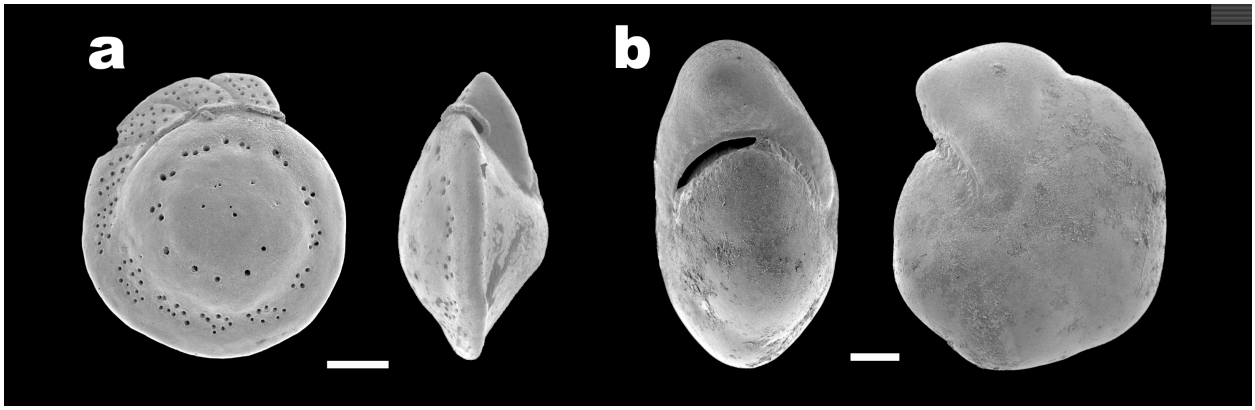

Figure 3.7. SEM picture showing the apertures of *Cibicides* (a) and *Pullenia* (b). Scale= 100μm

cibicidids and these two genera. *Cibicides* has the same kind of aperture as *Melonis* and *Pullenia*: a low interiomarginal slit with a lip (Fig. 3.7). Moreover, morphological intermediates (*Anomalina* d'Orbigny, 1826, *Anomalinoides* Brotzen, 1942) exist between the planispiral coil of *Melonis* and *Pullenia* and the trochospiral one of *Cibicides*, suggesting that a transition is possible. Therefore, the grouping of *Melonis*, *Pullenia* and *Cibicides* within the same family seems justified.

3.4.2. Relationships between the cibicidid species

Molecular data allow the distinction of three main clades within the cibicidids: clade 1 comprising Antarctic and Mediterranean populations of *C. refulgens*; clade 2 comprising *C. pachyderma* and *C. kullenbergi*, and clade 3, which includes *C. ungerianus*, *Cibicides* sp., *C. wuellerstorfi* and *C. lobatulus*.

Molecular distinction of three clades of cibicidids contradicts the traditional taxonomic separation between *Cibicides* and *Cibicidoides*, based on test convexity. The planoconvex species *C. lobatulus*, *C. refulgens* and *C. wuellerstorfi* form two separate clades, one of which also includes the planoconvex to slightly biconvex *C. ungerianus*. The two biconvex species, *C. kullenbergi* and *C. pachyderma* form a clade, which branches closer to *C. lobatulus*, *C. wuellerstorfi* and *C. ungerianus* than to *C. refulgens*. This is in agreement with critical remarks of some authors who already noticed that the separation between plano- and biconvex forms was not always clear and that both forms occurred within the same species (Mead, 1985; Verhallen, 1991; Gupta, 1994). Our results show that this distinction is not taxonomically relevant and confirm that the plano- or biconvex shape depends on the mode of life of the specimen, explaining why this can vary within one species.

3.4.3. Species identification

Among the six studied species, only *C. wuellerstorfi* appears to be well characterized genetically and morphologically. In all examined specimens the SSU sequences are almost identical (91-99%). The genetic homogeneity of this species is also confirmed by analysis of the much more variable ITS sequences (Pawlowski et al., work in progress).

Closely related to *C. wuellerstorfi,* is *C. lobatulus*. This species is also well characterized genetically, but its morphology is much more variable. It is often difficult to distinguish *C. lobatulus* from *C. refulgens*, especially when both species are found at the same localities and in similar environments (e.g. the specimens C170 to C173, sampled at the same location in the Mediterranean). *Cibicides refulgens* is often included within *C. lobatulus* in (paleo)ecological studies (see for instance Hageman, 1979; Verhallen, 1991), because of the morphological similarity and the observation of intermediate forms between both species (Verhoeve, 1971; Hageman, 1979; Van der Zwaan, 1982; Verhallen, 1991; Jonkers et al., 2002). *Cibicides lobatulus* comprises a huge variety of morphotypes which were sometimes described as different subspecies or even different species (Wood & Haynes, 1957; Nyholm, 1961; Cooper, 1965; Schnitker, 1969). Some specimens adopt strange shapes commanded by the substrate on which they live fixed; others, vagile, have a more regular shape. The molecular analyses show that regular (C120) and irregular (C35, C37) morphotypes branch together (Fig. 3.6), confirming that the large phenotypic variation within *C. lobatulus* is not phylogenetically relevant. On the other hand, a clear geographical separation between the population of *C. lobatulus* from the Mediteranean (C170) and the populations from the North Atlantic (C2) and the Skagerrak (576, C35, C37, C39, C40, C120) suggest that this species may comprise several cryptic species (Table 3.1; Fig. 3.6).

Cryptic speciation is evident in the case of *C. refulgens*. This species splits into two clades, one grouping the specimens from the Mediterranean, living attached to seaweeds, the second grouping the specimens collected in Antarctica. The latter live attached to the scallop *Adamussium colbecki* Smith, 1902 and feed on diatoms or on the mantle of their host, and can therefore be considered as parasites or predators (Alexander & DeLaca, 1987). Consequently, on the basis of these ecological and molecular differences, both populations should be considered as separate cryptic species, even if no morphological features can distinguish them yet.

Among the remaining four cibicidids, *C. pachyderma* and *C. kullenbergi* form a single clade, and

apparently belong to the same species. They are morphologically rather close and intermediates were observed between them (see Chapter 5). This implies that the name *C. pachyderma* should be retained for this morphospecies, while *C. kullenbergi* should be considered as its junior synonym. However, discrepancies in the species concept of *C. kullenbergi* exist and further sampling of other specimens is needed to confirm this synonymy. *Cibicides* sp. and *C. ungerianus*, are each represented by a single sequence and branch as sister groups to *C. wuellerstorfi*. *Cibicides ungerianus* appears distinct from *C. pachyderma* and *C. kullenbergi* contrary to the inference of Jonkers (1984) or Van Morkhoven et al. (1986).

3.5. Conclusions

As we have seen, current classifications have split the cibicidids into different genera, families and even superfamilies despite their common morphological and ecological features. Our study clearly shows that there is no justification for classifying the cibicidids in different superfamilies. According to our data, the planoconvex (*C. lobatulus, C. refulgens, C. ungerianus, C. wuellerstorfi*) and biconvex (*C. kullenbergi, C. pachyderma*) species group together suggesting that there is no reason to separate the biconvex from the planoconvex tests in two different genera (*Cibicides* and *Cibicidoides*), nor to split *Cibicides* into *Fontbotia* and *Lobatula* or to place *wuellerstorfi* in the genus *Planulina*. It seems justified to include all these species into the same family, and, for the time being, in the same genus *Cibicides* de Monfort, 1808. However, the monophyly of this genus should be investigated by more extensive taxon sampling and further analyses of other genes.
Within the genus *Cibicides*, some morphospecies have been confirmed by molecular analyses (*C. lobatulus*, *C. wuellerstorfi*), whereas others are probably different morphotypes of the same species (*C. pachyderma* and *C. kullenbergi*) or represent several cryptic species (*C. refulgens*). The morphological distinction between *C. lobatulus* and *C. refulgens* needs to be studied in more detail and their morphological definition should be revised. Samples from other localities around the world are clearly needed to test the species definition in widely distributed cibicidids and to fully answer all the questions addressed in this paper.