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Phylogenetic relationships among species of Glabratellidae (Foraminifera) inferred from ribosomal DNA sequences: Comparison with morphological and reproductive data

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INTRODUCTION

Foraminiferal genera and species have long been classified primarily using morphological characters of their tests. However, morphology of a foraminiferal test may sometimes be influenced by the ambient environment. It is, thus, difficult to determine which factors, genetic or ecophenotypic, mainly control morphological variations in foraminifera. Moreover, some foraminiferal species change morphology during ontogeny and/or in different phase of reproduction. For example, some species have been found to be simple reproductive stages of the same species (Nyholm 1961). Therefore, morphologically-based identification may be inadequate and inappropriate for estimating phylogenies.

During the past five years, several phylogenetic studies have been carried out on foraminifera using nucleotide sequences from both the small (SSU) and the large (LSU) subunits of ribosomal DNA (rDNA). This molecular method provided a new insight into foraminiferal phylogeny and allowed to revise the morphology-based classifications. For instance, Pawlowski et al. (1994) and Pawlowski et al. (1996) examined the phylogenetic position of foraminifera among eukaryotes, and showed that foraminifera branch deep in the eukaryotic tree. Darling et al. (1997) and de Vargas et al. (1997) investigated molecular phylogeny of planktonic foraminifera and suggested that planktonic foraminifera are polyphyletic. Their analysis indicated that at least two lineages of planktonic foraminifera exist today, both originated independently from benthic foraminifera. Darling et al. (1997) and Huber et al. (1997) showed also that there exist two genetic variants within a single morphospecies *Globigerinella siphonifera*, considered as cryptic species.

Before the molecular data are applied for inferring the phylogenetic relationships between foraminiferal species, however, it is necessary to examine genetic variation within morphological species or populations. High intraspecific sequence divergence can bias the results of phylogenetic analysis. It is well known that the rDNA copies can vary within a single specimen in benthic foraminifera. For instance, Holzmann et al. (1996) have found a high genetic variation, up to 5.2%, within a single specimen of *Ammonia* species. In this study, we have been concerned with how to interpret species using high sequence variation of ribosomal genes among the *Glabratella* group.

We chose to study the natural history of glabratellid genera (Kitazato 1988, Kitazato 1994), because species of these genera are relatively easy to culture. It is also easy to determine breeding abilities between individuals through breeding experiments, as glabratellid species make a plastogamic pair during sexual reproduction. The reproduction and morphological variations in glabratellid species, *Glabratella patelliformis*, *Planoglabratella opercularis*, and *P. nakamurai*, have been extensively examined and their classification was revised with respect to their morphological characteristics through ontogeny and between life-cycle generations (Kitazato et al. 2000). Both precise observations by SEM and interbreeding experiments show that morphogroups are consistent with same morphological characters. Each morphogroup can interbreed only within itself (Kitazato et al. 2000).

In this study, we examined the phylogenetic relationships among five species of Glabratellidae on the basis of both SSU and LSU rDNA sequence data. The main objective of this study is to test whether the morphology of Glabratellidae is controlled ecologically or genetically as both phenomena have been well documented (Kitazato et al. 2000). We studied phylogenetic relationships among species of glabratellids according to several different approaches: (1) we inferred phylogenetic relationships among species of Glabratellidae from LSU and SSU ribosomal DNA sequences, (2) compared between patterns of molecular phylogeny and ecology, such as morphology and breeding abilities, (3) discussed how ecological and morphological features evolved along the phylogenetic tree, and (4) then tried to explain the evolutionary trends among closely related species.

MATERIALS AND METHODS

Sampling and Identification

Living specimens of Glabratellidae were isolated from coralline algae collected from tide pools of rocky shores at ten localities along the Japanese Islands (text-fig. 1). Five species of Glabratellidae, *Glabratella milletti* (Wright), *G. patelliformis* (Brady), *Planoglabratella opercularis* (d'Orbigny), *P. nakamurai* (Asano), and *Angulodiscorbis quadrangularis* Uchio (text-fig. 2) were selected for this study. These species are common around the Japanese Islands.

Foraminifera were maintained at 15°C in petri dishes filled with autoclaved sterilized sea water (35‰) filtered with a 0.2μm membrane filter. Diatom cells, *Navicula* sp., were supplied as food. The diatoms were collected from tide pools of Omaezaki Cape and cultured at our laboratory in Shizuoka.

The specimens were brushed in 0.2μm filtered and autoclaved sterilized sea water with a thin writing brush before extraction

TEXT-FIGURE 1
Map of sampling localities indicating where five species of Glabratellidae were collected.

to remove any associated microorganisms. Specimens were then dried at room temperature for 15 minutes (Holzmann and Pawlowski 1996). Photographs of some extracted specimens were taken using a scanning electron microscope (JEOL-5600LV) under low vacuum condition (30Pa, 15kV) without any coatings in order to compare DNA data and morphological characters.

DNA extraction

Total DNA was extracted from all specimens one by one. Each specimen was transferred into a 1.5ml microfuge tube with sterilized sea water. The sea water was replaced with 50µl of TE buffer in the tube. The specimen was crushed with a siliconized and closed Pasteur pipette. Total DNA was extracted by the CTAB (Clark 1992) and the DOC (Pawlowski et al. 1994) methods and stored at -20°C.

PCR amplification

DNA fragments of about 700 base pairs (bp) situated at the 5' terminal region of the LSU rDNA, and of about 1,000 bp situated at the 3' terminal region of the SSU rDNA were amplified by polymerase chain reaction (PCR) using synthesized primer pairs. Primer pairs of Rib-2TA (5'-CACATCAGCTCGAGTGAG) and Rib-1F (5'-ACTCTCTCTTTCACTCC) were used for LSU amplification. Primer pairs of s14f1 (5'-AAGGGCACCACAAGAACGC) and sB (5'-TGATCCTTCTGCAGGTTCACCTAC) were used for SSU amplification. Both of those primer pairs were designed by Pawlowski et al. (1994) and Pawlowski et al. (1996). PCR amplifications were performed using 30 cycles with following conditions: denaturation; 94°C, 1 min., annealing; 55°C, 1 min., and extension; 72°C, 2 min. PCR products were purified with Spin-Bind DNA extraction units (FMC) and stored at -20°C.

Cloning and Sequencing

The purified PCR products were ligated in the pGEM-T vector System (Promega) and cloned into XL-2 blue ultracompetent cells (Stratagene). The DNA fragments were sequenced using fmol DNA Sequencing System (Promega) and the [γ-^{32}P] ATP end labeled primers. In addition, we used Hitachi SQ-5500 DNA sequencer and Thermo Sequenase pre-mix cycle sequencing kit (Amersham) with Texas Red labeled primers. For LSU analysis, we sequenced two clones per specimen for 35 specimens. We also sequenced five clones per specimen of both agamont and gamont for *G. patelliformis* to estimate sequence variations within one specimen. For SSU analysis, we sequenced 1 specimens for each morphospecies.

Sequence analysis

The rDNA sequences were aligned by using ClustalW (Thompson, Higgins and Gibson 1994) and corrected manually. Estimations of genetic divergence were calculated by the two-parameter method of correction of multiple substitutions at a site (Kimura 1980) (transition / transversions ratio = 2). The unrooted trees were constructed by neighbor joining (NJ) (Saitou and Nei 1987) performed by using ClustalW, and maximum parsimony (MP) using PAUP version 3.1.1 (Swofford 1993) with a branch-and-bound algorithm. Bootstrap was conducted with 1,000 replicates (NJ) and 100 replicates (MP). The sequences were deposited in the GenBank data base (accession numbers AF194033 - AF194078).

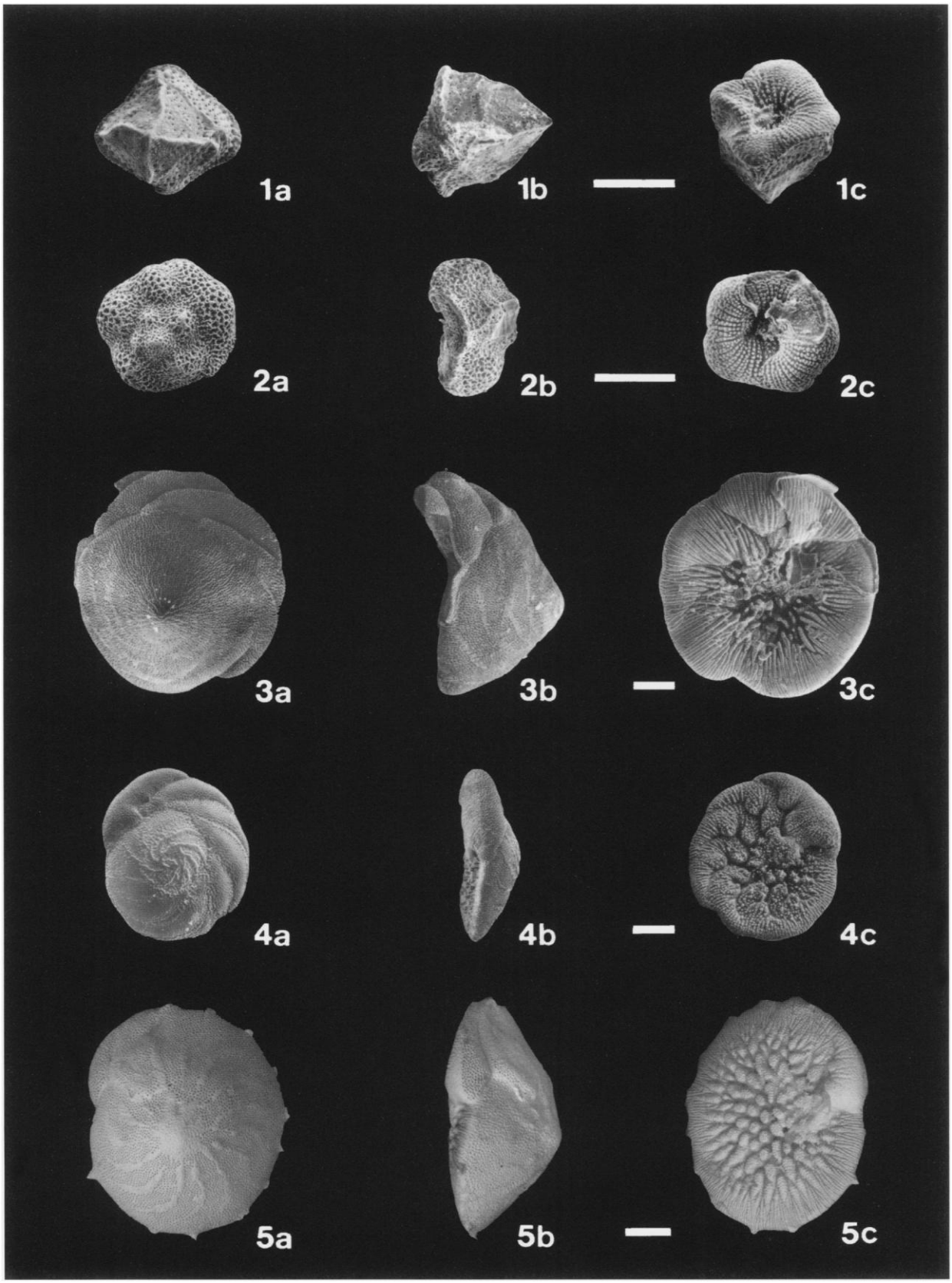
Rotaliella heterokaryotica was used as an outgroup for LSU analysis. This species is closely related to Glabratellidae according to morphological studies (Pawlowski and Lee 1991, Pawlowski and Lee 1992). The LSU rDNA sequence of *Glabratella mediterranensis* (GenBank accession number Z30149) collected from the Mediterranean Sea (Pawlowski et al. 1994) was added to analysis. On the other hand, *Buliminoides* sp., which also belong to Glabratellacea (Loeblich and Tappan 1988), was used as an outgroup for SSU analysis.

RESULTS

Position and length of amplified PCR fragments

The amplified LSU rDNA fragment in *P. opercularis* and other Glabratellidae corresponds to the position 6 - 634 bp of 5' terminal region of *Ammonia beccarii* (accession number X84304). This region includes the conserved domain C1 and C2, and divergent domain D1 (Hassouna et al. 1984). The position of SSU rDNA sequences of glabratellid species correspond to the 3' terminal region of the SSU, the same that has been examined by Darling et al. (1996) and Pawlowski et al. (1996).

The length of the LSU rDNA fragment varies from 533 to 577 bp between different morphospecies of Glabratellidae (Table 1a). The sequences are of almost the same length within all morphospecies, except for *P. opercularis*, in which the length variation up to 14 bp is observed between different specimens. In comparison, the amplified fragments of the SSU rDNA are nearly the same length for all morphospecies of Glabratellidae (Table 1b).



TEXT-FIGURE 2
SEM photographs of the five examined foraminifera. 1. *Angulodiscorbis quadrangularis* Uchio, 2. *Glabratella milletti* (Wright), 3. *Glabratella patelliformis* (Brady), 4. *Planoglabratella opercularis* (d'Orbigny), 5. *Planoglabratella nakamurai* (Asano), a. dorsal view, b. side view, c. ventral view. Scale bar indicates 100μm.

TABLE 1
Length variation and G+C content of both LSU and SSU rDNA sequences. a. Large subunit. b. Small subunit.

a	Large subunit (2TA-1F)	Length variation (bp)	G+C content (%)
	Angulodiscorbis quadrangularis	535-539	40.8
	Planoglabratella nakamurai	559-562	39.3
	Planoglabratella opercularis	548-562	39.6
	Glabratella milletti	533-546	39.4
	Glbaratella patelliformis	574-577	39.1

b	Small subunit (s14f1-sB)	Length variation (bp)	G+C content (%)
	Angulodiscorbis quadrangularis	1,001	44.1
	Planoglabratella nakamurai	1,003	44.3
	Planoglabratella opercularis	1,004	43.9
	Glabratella milletti	1,009	44.0
	Glbaratella patelliformis	1,002	43.0

In both molecules, the mean G+C content was similar among glabratellid species and ranged from 39.1 to 40.8% in the LSU, and from 43.0 to 44.3% in the SSU (Table 1a, 1b).

Sequence divergence

The genetic distances between glabratellid species range from 1.1 to 6.4% in the LSU and from 3.8 to 6.7% in the SSU (Tables 2a, 2b). The genetic distances between *P. opercularis* and *P. nakamurai* are smaller than between other species, ranging from 1.1% to 2.5% in the LSU and averaging 0.8% in the SSU.

The genetic distances within each morphospecies are lower than 1.1%, except for *P. opercularis*. In this species, the intraspecific variations reach up to 2.1%. This is due to the presence of two genetically different populations. In fact, two specimens of *P. opercularis* that were collected from the Oura Cove have different sequences from other populations of *P. opercularis* (Table 2a). The patterns of base substitutions in these two specimens (GO6 and GO170) are similar to those of *P. nakamurai* (text-fig.3a). Common nucleotide substitutions consist of 3 transitions (2 C-T transitions and 1 A-G transition), 8 transversions (4 A-T transversion and 4 C-A transversion), and 1 insertion/deletion.

Excepting two variants from the Oura population of *P. opercularis*, all other populations of this species are genetically similar. The specimens from closely located populations show very similar sequences (0-0.9%). Genetic distances are slightly larger in distantly separated populations, ranging from 0.5 to 1.1% between Omaezaki and Echizen-Matsushima. However, these dissimilarities among distant populations of *P. opercularis* are almost the same as those observed within an agamont of *G. patelliformis*.

The sequence divergence between the rDNA copies within the same specimen was examined in *G. patelliformis* (Table 2a). Small sequence differences are found among clones (text-fig. 3b). Nucleotide substitutions consist of 9 transitions (4 C-T transitions and 5 A-G transitions) and 3 transversions (1 C-A transversion and 2 G-T transversion). Agamonts of *G. patelliformis* show slightly larger (up to 1.1%) genetic variations than gamonts (up to 0.6 %).

Phylogenetic trees

Phylogenetic trees were reconstructed using both neighbor joining (NJ) and maximum parsimony (MP) (text-fig.4). The branching patterns were almost the same in LSU (text-fig. 4a, b) and SSU (text-fig.4c, d) trees. Specimens from the same morphospecies clustered together, excepting for two variants of *P. opercularis* from Oura which clustered with *P. nakamurai* in both NJ and MP analysis.

The branching patterns inferred from both LSU and SSU sequences indicate that *Glabratella* species diverged first, followed by the divergence of *Angulodiscorbis* and both *Planoglabratella* species. The branching order between the early diverging Glabratellidae is not well supported.

In all analysis, *P. opercularis* and *P. nakamurai* form a clade, however, this clade is well supported (100% bootstrap value) only in the SSU tree. The analysis of the LSU shows a close relationship between *Planoglabratella* and *Angulodiscorbis*; the latter genus groups with *P. nakamurai* in MP analysis.

DISCUSSION

Molecular data and fossil record

Analysis of SSU and LSU rDNA, using different species as outgroups, shows clearly that the family Glabratellidae is monophyletic. The branching order between examined species is relatively well established.

The analysis of both ribosomal genes gives congruent information concerning the respective positions of examined genera. In all trees, *Glabratella* branches first, followed by *Planoglabratella*, as shown in text-fig. 4. This topology corresponds to the fossil data, reflecting the order of apparition of these genera in the fossil record. According to Loeblich and Tappan (1988), *Glabratella* appeared in the early Miocene, while *Planoglabratella* appeared in the middle Miocene.

There is also a good agreement in relationship between two species of *Planoglabratella* and their fossil record. The first fossil appearance of *P. opercularis* in the Japanese Islands was dated at 15 Ma (Matoba et al. 1990), while *P. nakamurai* appeared about 9 Ma (Asano 1951). A presence of two different genotypes in Oura population of *P. opercularis* suggests, however, that the diversity in this genus may be higher than expected from the morphological study. Two specimens of *P. opercularis*, who are morphologically identical to other specimens of this species, but genetically more closely related to *P. nakamurai*, may represent in fact an evolutionary link between both species. Further study is necessary to examine if this is an isolated population which can be found only in Oura or this population is present also in other localities where *P. nakamurai* was found.

The only disagreement between molecular and fossil data concerns the position of *Angulodiscorbis*. This genus seems to have appeared in the Holocene (Uchio 1951), based on the fact that no Pleistocene fossils have been reported for this species. This contrasts with results of the molecular phylogeny suggesting more ancient origin of this genus. Two explanations are available for explaining these differences between molecular and fossil data. (1) One possibility is that pre-Holocene absence of *Angulodiscorbis* from the fossil record is an artifact of a poor

TABLE 2
Genetic distances of both LSU and SSU rDNA. a. Large subunit. b. Small subunit. *1: Genetic distance of two specimens from the Oura population; *2: Genetic distances among populations of *P. opercularis* without specimens of the Oura population; *3: Genetic distances for agamont (GP7) within a single specimen of *G. patelliformis*; *4: Genetic distances for gamont (GP19) within a single specimen of *G. patelliformis*.

a

Large subunit	*A. quadrangularis*	*P. nakamurai*	*P. opercularis*	*G. milletti*	*G. patelliformis*
Angulodiscorbis quadrangularis	0.2-0.9	***	***	***	***
Planoglabratella nakamurai	1.6-2.5	0-0.9	***	***	***
Planoglabratella opercularis	1.6-2.8	1.1-2.5	0-2.1 (0.7)*1; (0-1.1)*2	***	***
Glabratella milletti	4.7-6.4	4.2-5.9	4.0-5.9	0-0.7	***
Glbaratella patelliformis	4.0-4.7	3.2-4.4	3.7-5.2	4.7-5.4	0-1.1 (0-1.1)*3; (0-0.6)*4
Rotaliella heterokarotica	18.4-19.3	18.7-19.3	18.4-19.3	18.7-19.0	19.0-20.4

b

Small subunit	*A. quadrangularis*	*P. nakamurai*	*P. opercularis*	*G. milletti*	*G. patelliformis*
Angulodiscorbis quadrangularis	***	***	***	***	***
Planoglabratella nakamurai	3.8	***	***	***	***
Planoglabratella opercularis	3.9	0.8	***	***	***
Glabratella milletti	6.1	6.7	6.7	***	***
Glbaratella patelliformis	5.2	5.1	5.5	6.7	***
Buliminoides sp.	15.4	15.9	15.9	15.9	15.9

fossil record. (2) Another possibility is that our results are biased by limited number of Glabratellidae species selected for this analysis. Five species of Glabratellidae used in this study present a small part of 18 genera belonging to this family according to Loeblich and Tappan (1988).

Angulodiscorbis is the only genus which position at the phylogenetic tree is not well established. This genus appears as a sister group to *Planoglabratella* in analysis of the SSU and in NJ analysis of the LSU, but it branches with *P. nakamurai* in the MP analysis of the LSU (text-fig.4b). This difference may be caused by the distant relationship between ingroup and outgroup species that influenced the tree topology and the bootstrap confidence. We cannot exclude that the phylogenetic tree of Glabratellidae will have a different branching pattern if we add another glabratellid for phylogenetic analysis. High bootstrap values were obtained when *Buliminoides* sp. was used as an outgroup in the SSU tree. This genus belongs to the family Buliminoididae which is considered as a sister group to the Glabratellidae (Loeblich and Tappan 1988). However, its LSU rDNA sequence could not be obtained. In order to ascertain the phylogeny of this group, further studies are necessary to analyse the fossil record and phylogenetic reconstruction using other species of Glabratellidae and related families.

Molecular data and test morphology

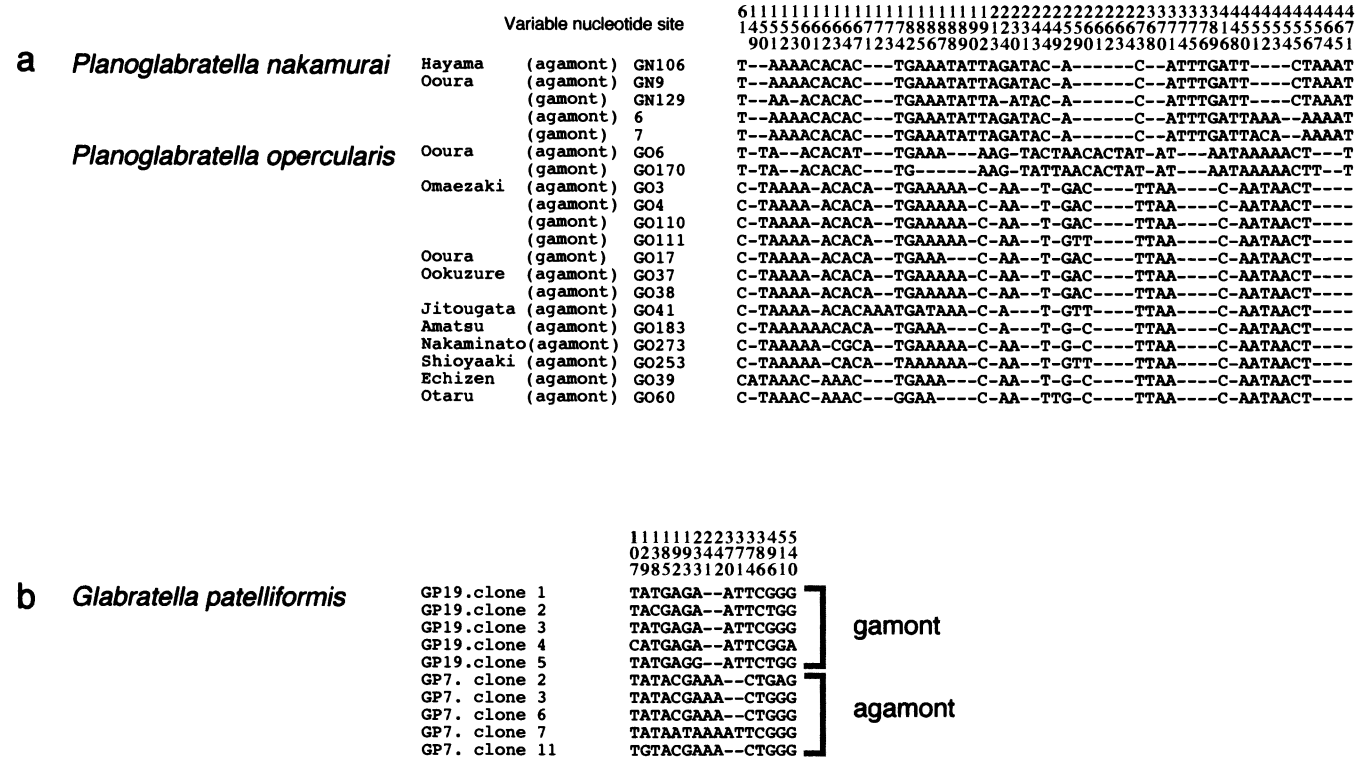
The examined species of Glabratellidae can be divided into two larger distinct morphogroups that possess specific morphological characters. One group is composed of two *Planoglabratella* species. The other includes both *Angulodiscorbis* and *Glabratella* species. Two *Planoglabratella* species have umbilical bosses on their ventral sides, while both *Angulodiscorbis* and *Glabratella* have straight striae lines on the ventral side (text-fig. 2). Except for ventral side sculpture, full-test morphological characters such as chamber shape, number of last whorl chambers, height, maximum test diameter and shape of dorsal side, differ from species to species.

Molecular data are congruent with the distinction of two morphogroups. The morphospecies that are closely related at the molecular phylogenetic tree possess the same ventral sculpture. Each branch of the molecular phylogenetic trees corresponds well to a specific morphospecies that was classified using morphological characters of the tests. The branching order of different morphospecies at the phylogenetic tree give us some information on the trends in morphological evolution of Glabratellidae. If we focus on ornamentation of the ventral side, we can found that the presence of straight striae and a central aperture, like in *Glabratella* and *Angulodiscorbis*, is ancestral compared to the short striae, central bosses and marginal aperture observed in *Planoglabratella* (text-fig. 2).

Our study confirms that the ventral side sculpture is an important morphological character for discriminating glabratellid species. This character is supposed to play a crucial role in reproduction of Glabratellidae (Kitazato 1992, Kitazato et al. 2000). Ventral sculpture is most probably related to rhizopodial activities involved in sexual reproduction, movement, and selection of food particles (Kitazato 1992). The changes of this character correspond to the long-term trends in morphological evolution and therefore they can be useful for morphological identification.

Molecular data and breeding ability

The Glabratellidae are one of the rare groups of foraminifera for which the breeding experiments have been successfully realized



TEXT-FIGURE 3
Variable nucleotide sites among LSU rDNA sequences of *Planoglabratella* species (text-fig. 3a) and *Glabratella patelliformis* (text-fig. 3b).

(Kitazato et al. accepted). These experiments clearly distinguish and morphologically characterize the breeding populations.

Each clade at the molecular phylogenetic tree corresponds to a morphospecies that has a potential breeding ability, according to the intra- and interspecific breeding experiments (Kitazato et al. 2000). The breeding is not possible between these morphospecies and therefore they can be considered as "true" species, in agreement with a biological species concept. Kitazato et al. (2000) suggested that these morphospecies may be the "ring species", because, geographically distant populations of *P. opercularis* do not breed between themselves, even though there is no LSU rDNA sequence differences between populations.

Intraspecific variations in *G. patelliformis*

It is relatively easy to recognize morphologically the gamont and agamont in Glabratellidae and therefore to examine the genetic variations between and within these life-cycle stages.

In this study, we have compared the LSU rDNA sequence divergence between and within the gamont and agamont of *Glabratella patelliformis*. The sequences of both generations cluster together confirming that two morphotypes represent a single species. However, the genetic distances between the clones obtained from an agamont are almost two times larger, in terms of percentage, than those observed between the clones from a gamont. If this difference is confirmed by sequencing of a larger number of specimens, that would suggest that the variation of rDNA copies are somehow related to the multiplication and differentiation of nuclei in agamont. Similar explanation was given for the genetic variation observed within some, but not all, individuals of *Ammonia* spp. (Holzmann et al. 1996). However in the case of *Ammonia*, the life-cycle stages have not been morphologically recognized.

Compared to the study by Holzmann et al. (1996), genetic variations within a single specimen of *Glabratella* are smaller than those observed in *Ammonia*. This may be due to the differences in rates of substitution between both genera. It is possible, however, that genetic variations may be related to differences in reproductive behavior between *Glabratella* and *Ammonia*. During sexual reproduction, two gamont individuals of *Glabratella* spp. attach to each other along their ventral sides and make a plastogamic pair. They exchange gametes within the tests. Juvenile agamont individuals with two or three chambers moved out from the plastogamic pair. This reproductive behavior shows that the breeding population size of a glabratellid may be small. In contrast, *Ammonia* which releases gametes into ambient waters, has much larger gamete dispersal ability than glabratellids. It is possible that the extent of breeding may be larger than that of glabratellids, because glabratellids have low gamete dispersal ability.

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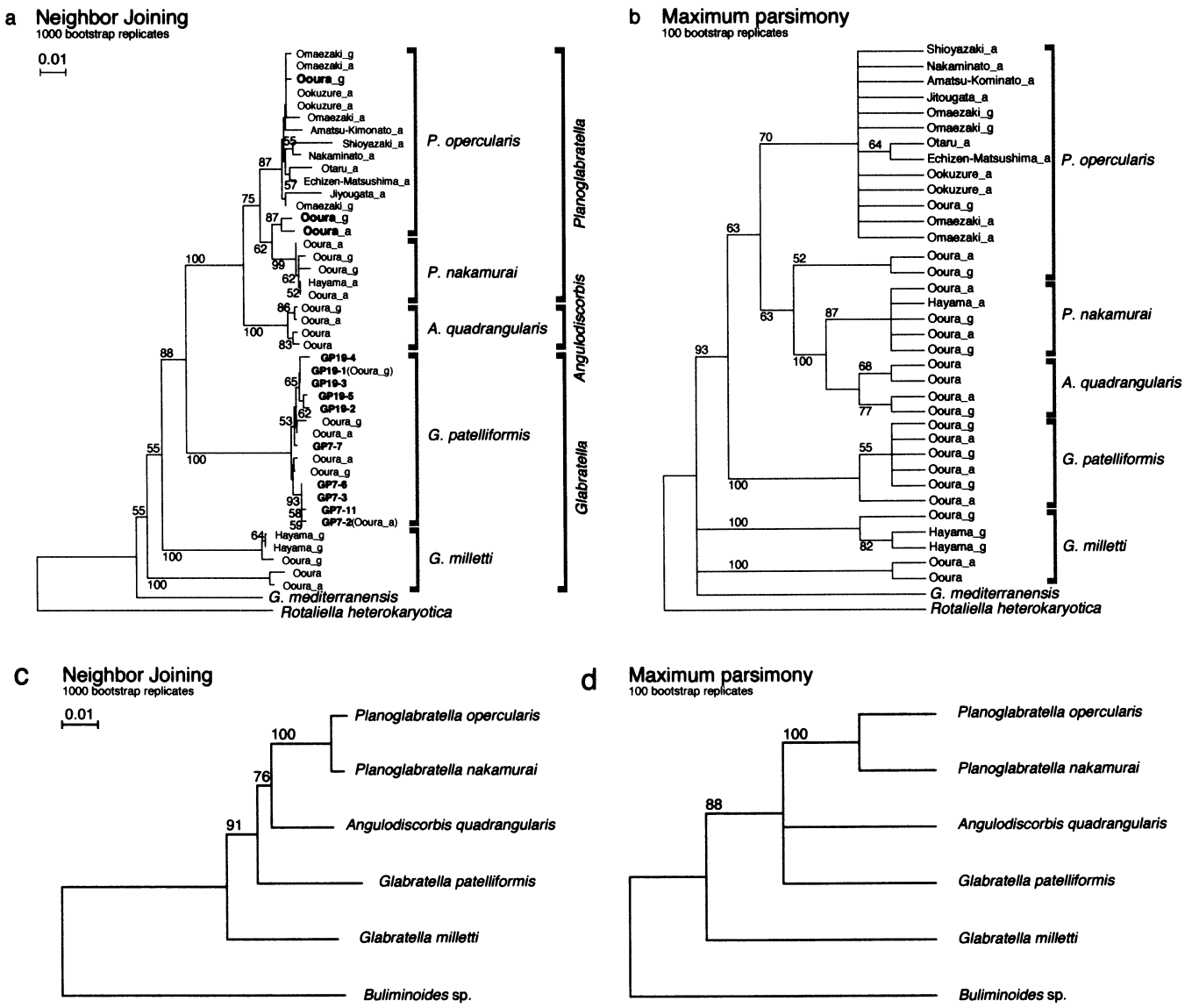

TEXT-FIGURE 4
Evolutionary relationships of LSU and SSU trees among five species of Glabratellidae. The unrooted trees were constructed by neighbor joining (NJ) (text-fig. 4a and 4c) and maximum parsimony (MP) (text-fig.4b and 4d). Bootstrap was conducted with 1,000 (NJ), and 100 (MP) replicates. Locality and generation (agamont=a, gamont=g) was given for each specimen. In text-figure 3a, five clones of gamont and agamont of *G. patelliformis* are shown as the specimen number, GP19 (gamont) and GP7 (agamont). Two genotypes of *P. opercularis* from the Oura population are indicated in bold.

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