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Genus *Gambierdiscus* in the Canary Islands (NE Atlantic Ocean) with Description of *Gambierdiscus silvae* sp. nov., a New Potentially Toxic Epiphytic Benthic Dinoflagellate



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Species of the dinoflagellate genus *Gambierdiscus* are the cause of Ciguatera Fish Poisoning, common in tropical areas. Nevertheless, until recently this syndrome was not reported in the NE Atlantic Ocean. A new photosynthetic dinoflagellate species, *Gambierdiscus silvae* sp. nov. is described based on samples taken from tide pools on rocky shores of the Canary Islands (NE Atlantic Ocean). Its morphology was studied by light and scanning electron microscopy. The new species is anterioposteriorly compressed, lenticular in shape with an epitheca slightly higher than the hypotheca. It is round in apical view and has a thick smooth theca with many scattered pores. Plate formula is Po, 4', 0a, 6'', 6c, 6s?, 5''', 0p, 2'''. Plate 2' is hatchet-shaped and Plate 2''' is very wide and the largest of the hypotheca. Phylogenies inferred from the large subunit nuclear rRNA showed that three *G. silvae* strains clustered in a well supported sister clade to *G. polynesiensis*, distinct from the other species. *G. australes* was observed for the first time in the Atlantic, together with *G. excentricus* already reported from these islands. This work increases the number of *Gambierdiscus* species described and shows their unexpected diversity in the Canary Islands.

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Key words: Benthic dinoflagellate; Canary Islands; *Gambierdiscus*; HABs.

Introduction

The genus *Gambierdiscus* was erected by Adachi and Fukuyo (1979) with *G. toxicus* as the type species of a monospecific genus. It was considered as a monospecific genus for many years

but up to 12 species were described (GEOHAB 2012; Hoppenrath et al. 2014; Nishimura et al. 2014; Parsons et al. 2012). Based on the information given by all these species, *G. toxicus* was re-described as it was observed that the original description was done involving more than one species (Litaker et al. 2009). In fact, it is quite common to observe several species of *Gambierdiscus* in the same area (GEOHAB 2012; Litaker et al. 2010; Nishimura et al. 2013; Parsons et al. 2012;

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Xu et al. 2014) and the morphological differences among them are very subtle making it difficult to recognize their diversity in a particular place.

Ciguatera fish poisoning is a syndrome caused by eating toxic marine fish from tropical and some temperate areas. It is caused by toxins produced by dinoflagellates of the genus *Gambierdiscus* which upon ingestion are metabolized, accumulated and transmitted through the food web to humans (GEOHAB 2012; Parsons et al. 2012). Ciguatera was considered as a tropical syndrome, although intoxications in temperate countries were caused by imported fish (Caillaud et al. 2010, and references therein). Recently, some cases of ciguatera were reported following the consumption of local fishes in temperate areas like the Canary Islands (Boada et al. 2010; Pérez-Arellano et al. 2005). *Gambierdiscus* species were reported from non-tropical seas only recently (Aligizaki and Nikolaidis 2008; Aligizaki et al. 2008; Fraga et al. 2011; Nishimura et al. 2013). Although the Eastern Atlantic Ocean was not considered an affected area, the oldest historical record of ciguatera was described from the Gulf of Guinea in 1521 (Urdaneta 1580), and the first world's report of an observation of cells of *Gambierdiscus* (although referred as *Goniodoma* sp.), dated from October 1948 near the coast of Boavista Island, in the Cabo Verde archipelago (Fig. 1) (Silva 1956).

In this study, new information on the presence of *Gambierdiscus* in the Canary Islands is given with the first report of *G. australes* in the Atlantic Ocean and the description of a new species, *Gambierdiscus silvae* sp. nov., on the basis of morphology. This species (formerly known as *G. sp. ribotype 1*) (Litaker et al. 2010), turned out to be morphologically different from the other species of *Gambierdiscus* already described and genetically different from other *Gambierdiscus* species and phylotypes. We suggest that *G. silvae* is probably the species reported by Silva (1956) from Cabo Verde archipelago as *Goniodoma* sp.

Results

Three *Gambierdiscus* species, *G. excentricus* S. Fraga, *G. australes* M. Chinain and M.A. Faust and *G. silvae* sp. nov. were found in the Canary Islands (Fig. 1). The three species were found in tide pools in rocky shores. *G. silvae* was first isolated from a sample taken in winter 2010 on the middle eulittoral zone in Gran Canaria, whereas the strains isolated from the 2013 samples were from the lower levels of the eulittoral zone in Tenerife (Fig. 1) where it

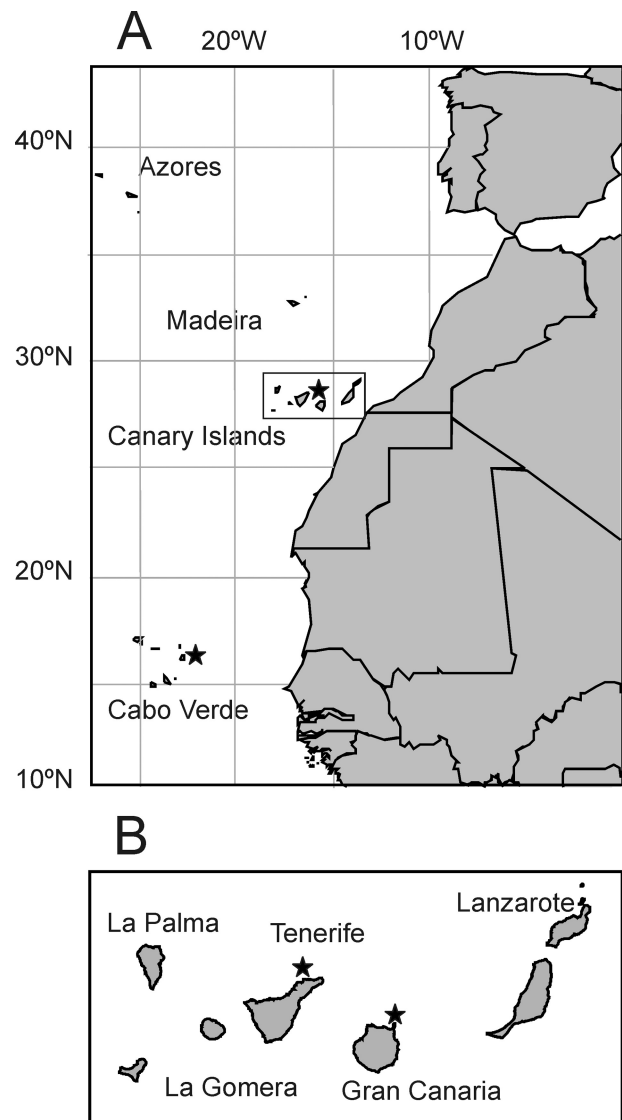


Figure 1. A) Map of the East Atlantic archipelagos. B) Map of the Canary Islands. Stars: Localities where *Gambierdiscus silvae* was found in Canary Islands and *Goniodoma* sp. in Cabo Verde.

coexisted with *G. excentricus* and *G. australes*. *G. australes* was only isolated from the 2013 sampling and it was found in tide pools from the lower to the upper levels of the eulittoral zone where the other two species were not observed. *G. excentricus* was observed, but not successfully cultured, in few samples obtained in lower level tide pools.

Morphology

Cells of *G. silvae* are anterioposteriorly compressed, lenticular in shape with the epitheca slightly taller than the hypotheca and symmetrical in ventral view (Figs 2C, 4C), $46 \pm 5 \mu\text{m}$ long,

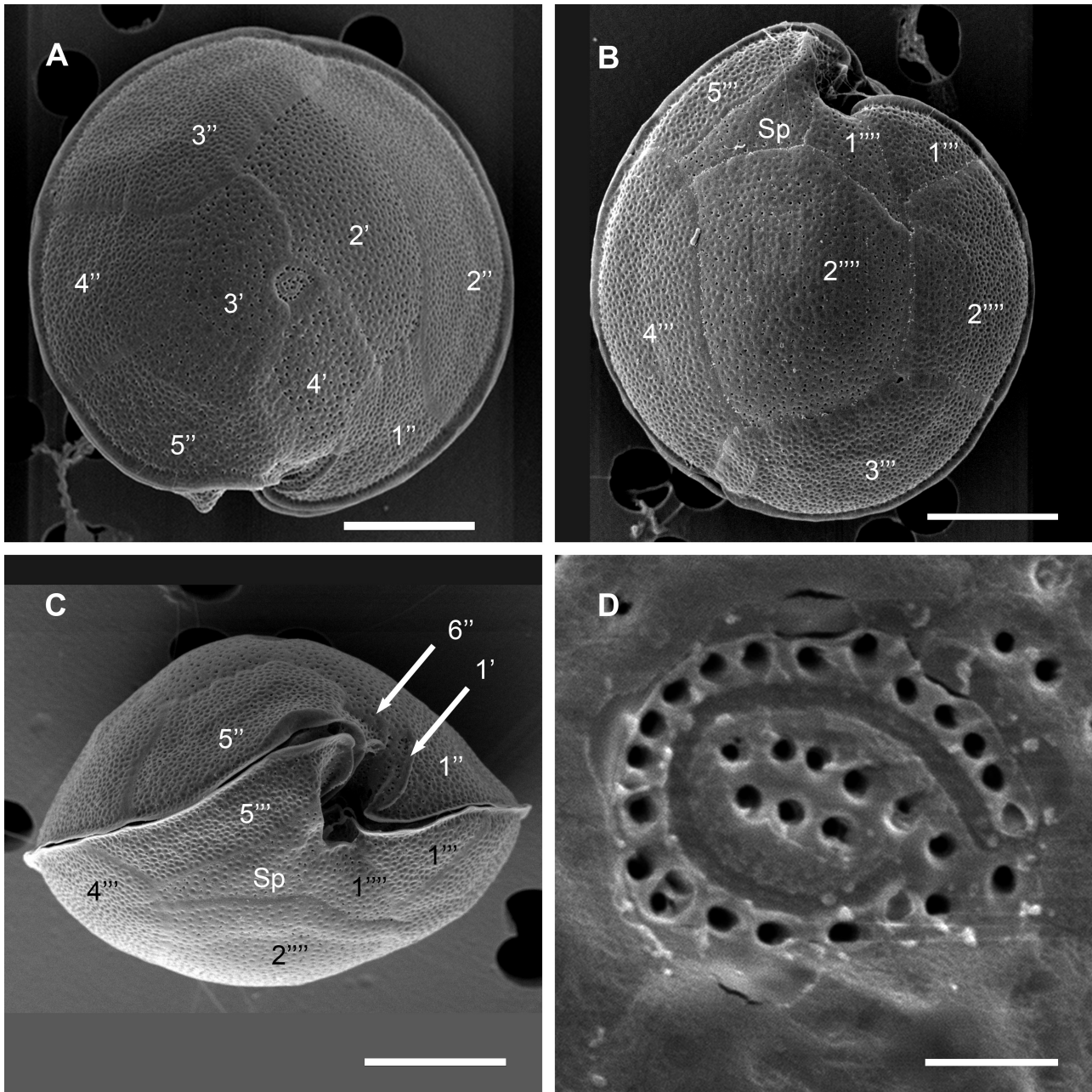


Figure 2. *Gambierdiscus silvae* (strain VGO1022). SEM images **A)** Apical view, **B)** Antapical view, **C)** Ventral view, **D)** Po plate. Scale bars: 20 μm , except D, 2 μm .

69 \pm 8 μm deep, 64 \pm 9 μm wide. It is round in apical and antapical view (Figs 2A, B, 3A, B, 4A, B) and has a thick theca with many scattered pores. Apical and antapical plates are smoother than pre- and postcingular plates that show a pattern of shallow depressions more visible near the cingulum (Figs 2A, B, 5A, 6, 7B). Pores in apical and antapical plates are larger and less numerous than in pre- and postcingular plates. For example,

in Plate 2'''' 0.28 μm \pm 0.04 (n=18) and Plate 4''' 0.16 μm \pm 0.03 (n=28) (Fig. 6). The plate formula is Po, 4', 0a, 6'', 6c, 6s?, 5''', 0p, 2'''''. The apical pore complex (Po) is oval, has a fish hook-shaped slit, and it is centred in the epitheca, only slightly ventrally displaced (Figs 2A, D, 3A, 5C, D, G). Plate 1' is small, narrow and pointed anteriorly (Fig. 5B, E), it is very small and does not contact Po. Plate 2' is hatchet-shaped and it is usually the largest of

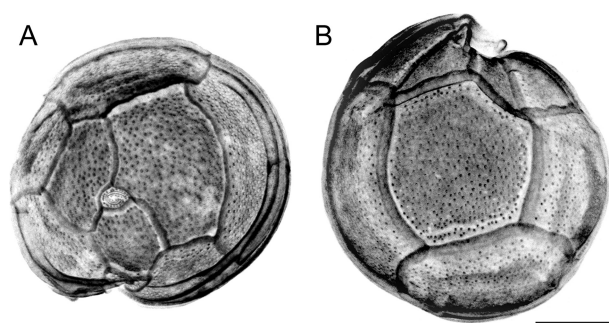


Figure 3. *Gambierdiscus silvae* (strain VGO1180). CLSM images of calcofluor stained thecae. **A)** Epitheca, **B)** Hypotheca. Scale bar 20 μm .

the epithelial plates (Figs 3A, 5B). In old cultures, where by chance, couples of fusing cells were observed, there were many tall cells, almost spherical with pre- and postcingular plates elongated in the apical axis, while apical and antapical plates have the same size as common cells (Fig. 5A, C, D). In these cells, Plates 2'' and 3'' are clearly bigger than 2'. In most of the cells, the sutures 2'/1'' and 2'/2'' do not form an angle as in Figures 3A and 5B but a continuous curve (Figs 2A, 5D, F, G) giving Plate 2' the shape of a pointed axe. The suture 2'/3'' is about twice as 2'/1''. Plate 3' is about same size as 4' (Figs 2A, 3A, 4A, 5B). Precingular plates have different sizes but 2'' is wider and can be the biggest of the epitheca in tall cells (Fig. 5A, C, D). Plate 3'' is asymmetrical having suture 3''/2'' longer than 3''/3' (Figs 2A, 3A, 4A, 5B). Plate 6'' is very small, and with 1', it forms the anterior edge of the sulcus (Figs 2C, 5B, 9A, B, D). Plates 1', 4' and 1'' do not form a distinct fold. The cingulum, which is very narrow and deeply excavated (Figs 2C, 9A, B), has six plates, and among them, c2 and c4 are the larger. Plate c1 is overlapped by c2, c3 overlaps c2 and c4, c4 overlaps c5, and c5 overlaps c6 (Figs 4B, 8). The sulcus is like a funnel with the longitudinal flagellum always radially orientated in the equatorial plane instead of being posteriorly orientated as in most dinoflagellates. The sulcus is very short and the Sp plate is outside of the sulcus (Figs 2B, C, 4B, C, 7A, B, 9B). The whole sulcal area is clockwise twisted in ventral view, so the Sa plate is displaced to the left side of the cell and the Sp to the right side. This gyre gives the impression of an ascending cingular displacement although it has a descending cingular displacement as in the other Gonyaulacales. From the ventral side, three parallel sulcal plates are easily observed from right to left: Sdp, Sda and Sa (Figs 8, 9). Plate Sdp is in contact to 5''' and with that plate it forms a wing on the anterior side of the sulcus (Figs 9A, B).

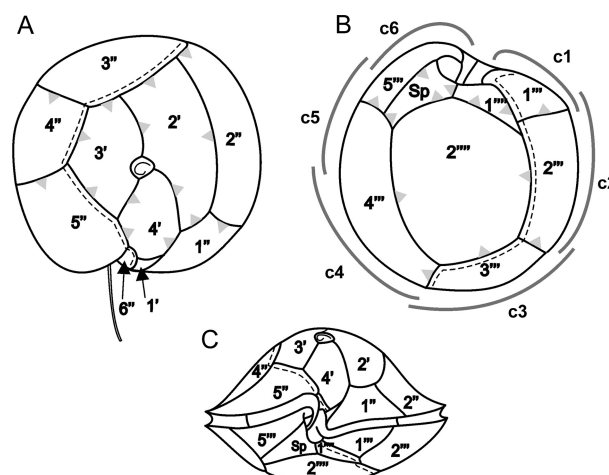


Figure 4. Ink drawings of *G. silvae* based on strain VGO1022. **A)** Apical view, **B)** Antapical view and cingular plates, and **C)** Ventral view. Dashed lines indicate fission line and triangles plates overlap. Plates 1' to 4', apical plates; Plates 1'' to 6'', precingular plates; plates c1 to c6, cingular plates; Plates 1''' to 5''', postcingular plates; Plates 1'''' to 2''', antapical plates; Plate Sp, sulcal posterior plate.

Plate Sda is like a prolongation of the cingulum contacting, at least, c6, 6'', Sa and Sdp (Fig. 9A). Plate Sa contacts 6'' and 1' in the anterior side, 1'' in the left side and penetrates into the deeper area of the funnel like sulcus where it contacts c1 and Ssa (Fig. 9). Ssa and Ssp are in the deepest part to the funnel and lie behind plates Sp, 1'''' and 1''' (Fig. 9B).

In the hypotheca, Plate 1''' is triangular, Plates 2''' and 3''' are four sided (Figs 2B, 3B, 4B). Plate 4''' can be five or four sided as the connection to Sp is variable (Figs 2B, 4B, 7B). Plate 5''' is narrow and near its ventral side it has a fold which together with the anterior side of Sp forms the edge of the sulcal funnel (Figs 7B, 9A, B). Plate 2'''' is very wide (Figs 2B, 3B, 4B, 7A-C, 8) and usually occupies most of the hypotheca in a way that all the other plates appear like compressed to the periphery (Figs 3B, 4B, 7A). Nevertheless, in tall cells the relative size of 2'''' is smaller compared as it is in common cells. Plate 1'''' usually does not contact 2'''' but sometimes these two plates plus 1'''' and 2''' meet at one point (data not shown). In a similar way, 2'''' is usually not in contact to 5''' (Figs 2B, 5A), but in some cases these two plates have a common suture, and Plate 2'''' is six sided instead of five sided (Fig. 7B).

The nucleus is usually arc-shaped with points ventrally directed (Fig. 10A). Cells of *G. silvae*

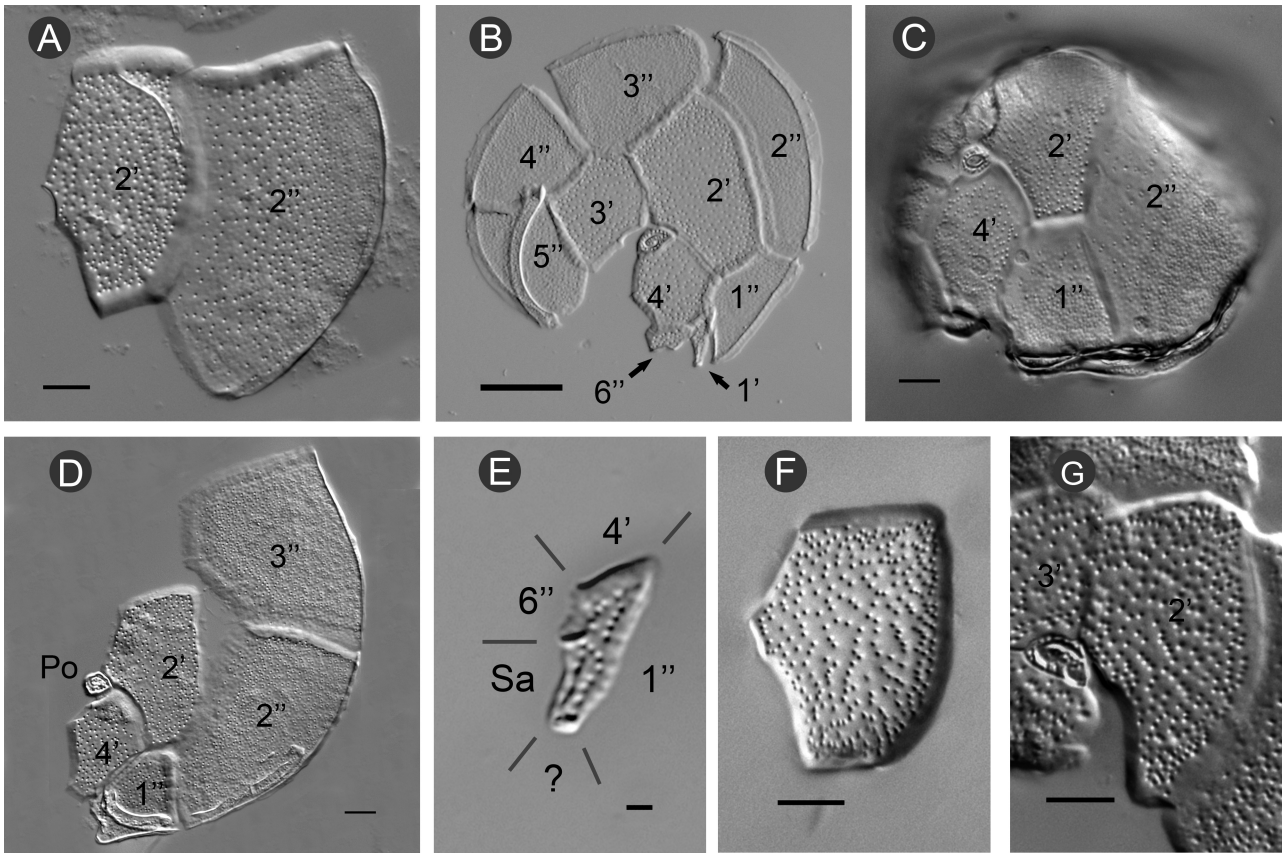


Figure 5. *Gambierdiscus silvae* strain VGO1022 (A, C, D, F and G) and strain VGO1180 (B, E). LM images of epithelial plates. **A)** Plates 2' and 2''. **B)** Dissected epithelial plates. **C)** Left side of the epitheca of a tall cell. **D)** Squashed epitheca of a tall cell with big precingular plates. **E)** Plate 1' 1' with indication of the position of neighbouring plates. **F-G)** Different examples of Plate 2'. Scale bars: 10 μm except B, 20 μm and E, 2 μm .

are photosynthetic and have numerous round and small chloroplasts (Fig. 10B).

The cells of *G. australes* matched the morphological characteristics of the species (Chinain et al. 1999; Litaker et al. 2009) namely, rectangular Plate

2', narrow 2''', asymmetric 3' and smooth surface (Fig. 11). The size of the *G. australes* cells from Tenerife have an average length $46 \pm 6 \mu\text{m}$, depth $85 \pm 8 \mu\text{m}$, and width $76 \pm 7 \mu\text{m}$.

Distribution

Gambierdiscus silvae was found associated with *G. excentricus*, *G. australes*, *Prorocentrum lima*, *Prorocentrum* spp., *Ostreopsis* spp., *Coolia* spp., *Bysmatrum* sp., *Amphidinium* sp. and *Heterocapsa* sp. as epiphytic on a mixture of small seaweeds in rocky tide-pools on the NW coasts of the islands of Gran Canaria and Tenerife. These areas were exposed to the dominant trade winds (e.g. the day of sampling in La Puntilla, winds over 120 Km h^{-1} were recorded). Seawater temperature varied from $18\text{-}26^\circ\text{C}$ where the yearly average of 21°C was reported (Borges et al. 2004). During the Punta Hidalgo sampling the seawater temperature was between $23\text{-}24^\circ\text{C}$.

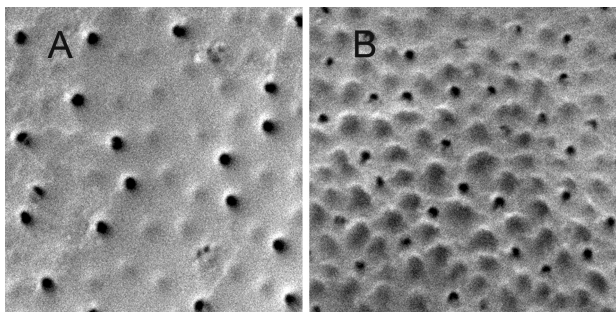


Figure 6. *Gambierdiscus silvae* (strain VGO1180). SEM images of two squares of $10 \times 10 \mu\text{m}$ ($100 \mu\text{m}^2$) of thecal surfaces of the same cell. **A)** Antapical Plate 2'''. **B)** Postcingular Plate 4'''.

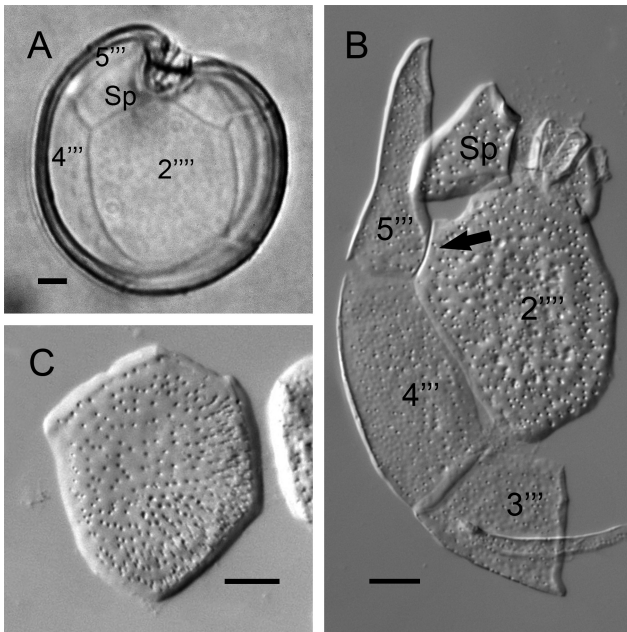


Figure 7. *Gambierdiscus silvae* (strain VGO1022). LM images of hypothecal plates. **A)** Whole hypotheca with a wide Plate 2'''' which is not in contact to Plate 5'''. **B)** Several dissected hypothecal plates showing 2'''' in contact to 5'''' (arrow). **C)** Plate 2'''' . 2'''' . Scale bars: 10 µm.

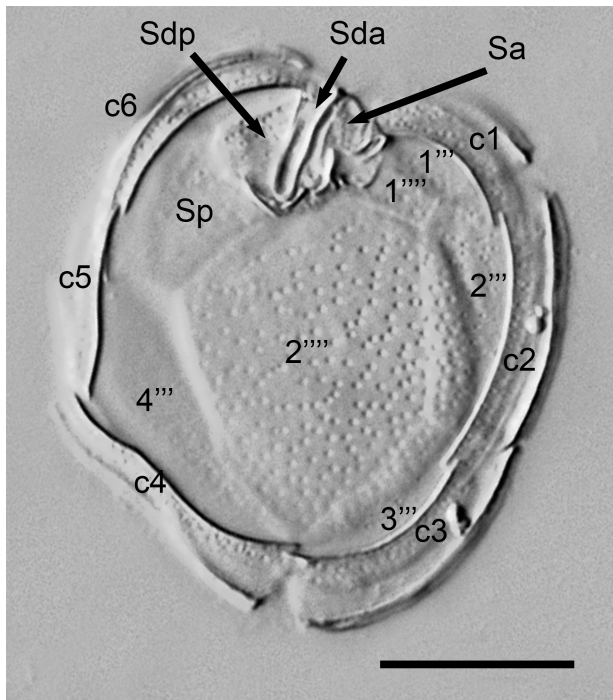


Figure 8. *Gambierdiscus silvae* (strain VGO1180). LM image of cingular plates in relation to hypothecal and sulcal plates. Scale bar: 20 µm.

Phylogenetic Analyses

Two phylogenetic trees based on D8-D10 and D1-D3 regions of the LSUrRNA gene were elaborated (Figs 12–13). Both phylogenies reconstructed the same relationships between clades of *Gambierdiscus* species (and ribotypes/sp. types not formally described as species). Sequences of *G. silvae* strains VGO1022, VGO1167 and VGO1180 clustered together as a separate group, sister to *G. polynesiensis*. In the D8-D10 phylogeny (Fig. 12) the alignment could include several sequences from Belize, labelled as *G. sp.* ribotype 1 by Litaker et al. (2010), which grouped together with *G. silvae* from the Canary Islands isolates. In the D1-D3 phylogeny (Fig. 13) the *G. silvae* clade was built only with the sequences from the Canaries isolates, as no other reports for this species could be retrieved from GenBank.

Discussion

Morphology

Gambierdiscus silvae has a hatchet-shaped Plate 2', an asymmetrical 3' and a broad 2''', as *G. polynesiensis* and *G. carolinianus*. It is very similar to *G. polynesiensis* in general shape and tabulation but it differs from it in the lack of the distinct fold formed by 4', 1' and 1'' in *G. polynesiensis* (named as 1', 1'' and 2'' in Litaker et al. 2009). The shape of Po, which is oval in *G. silvae* and triangular in *G. polynesiensis*, while in ventral view *G. silvae* is almost symmetrical compared to *G. polynesiensis* and has also different plate textures. *G. silvae* differs from *G. carolinianus* in the shape of 1' which in *G. carolinianus* is shorter than in *G. silvae*. *G. carolinianus* is also wider than deep while *G. silvae* is deeper than wide. *Gambierdiscus* sp. type 4 as described by Xu et al. (2014) looks like *G. silvae* in their figures 5A and C by the shape of 2' and the difference in texture between apical and precingular plates, but the hypotheca looks different as Plate 2'''' (their 1p) looks not as wide as in *G. silvae*.

The species reported by Silva (1956) as *Goniodoma* sp. (Fig. 14) was probably *G. silvae*. No differences can be observed in the epitheca or in ventral view. The drawing of the hypotheca is more difficult to interpret as, although it is not optically reversed, it is observed from inside as in figures 4B and 4C in Nishimura et al. (2014) in a way that cingular and some sulcal plates are overlapped to the hypothecal plates and hence, hiding Plates 5'''' and 1'''''. Plate 2' is hatchet-shaped and the sutures with 1'' and 2'' are in a continuous curved line. Plate 2'''' is wide.

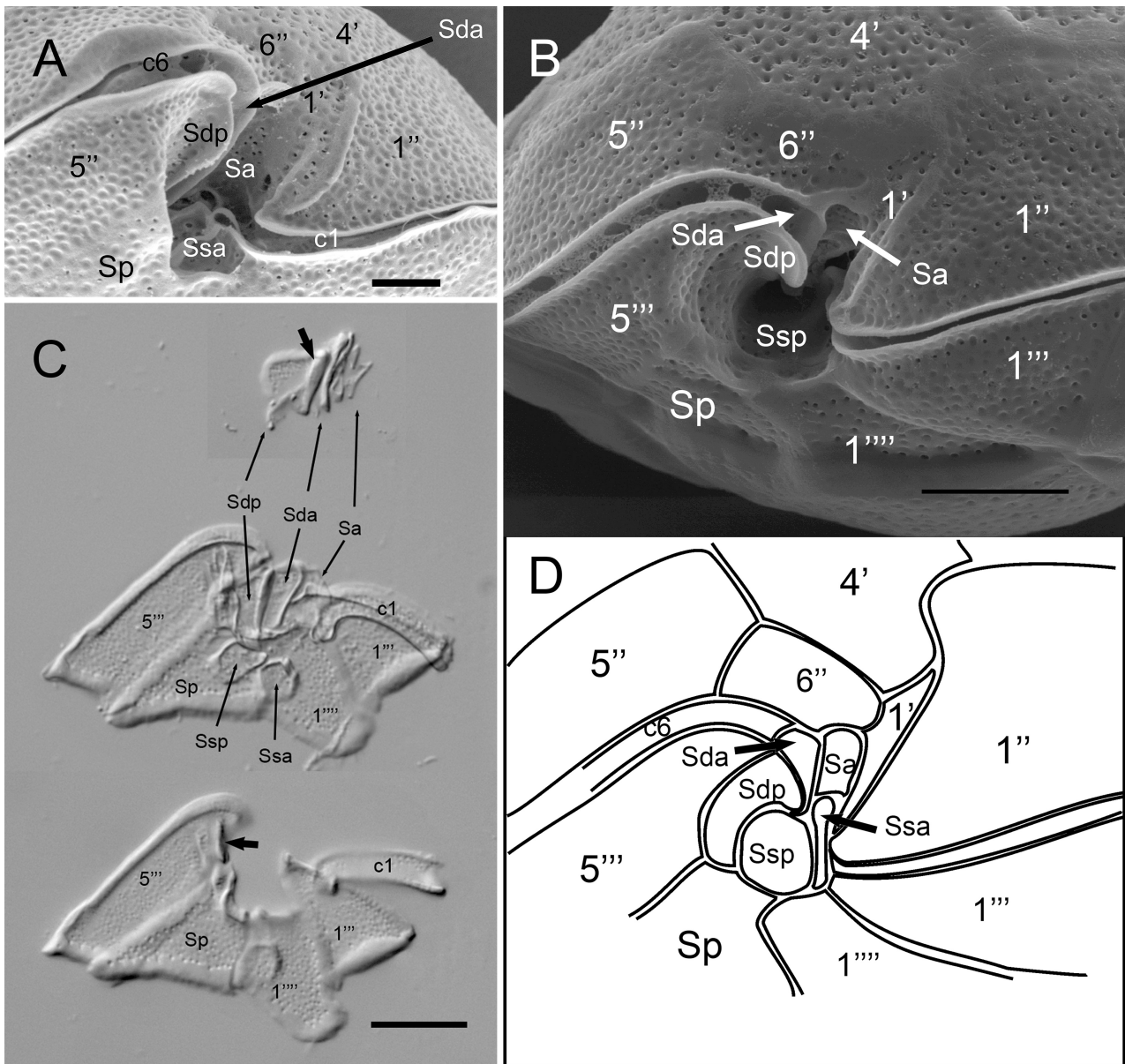


Figure 9. *Gambierdiscus silvae* (strain VGO1180). SEM and LM images of sulcal plates in relation to neighbour plates. **A)** SEM image of sulcus in ventral right view. Plate Ssp is not visible in this view as it is behind Sp. **B)** SEM image of sulcal area of other cell in different orientation. **C)** LM images of dissected sulcal plates. In the middle of the figure, the whole set of plates is shown, while on top and bottom the same plates are shown after more dissection. In the middle figure plates Ssp and Ssd, which were in different focus, were overlapped to plates Sp and 1''''', respectively. A thick arrow in the top figure points to the wing of the left side of Sdp. The thick arrow on the bottom figure shows the ridge in Plate 5''''', which is part of the edge of the sulcal hollow together with the anterior sides of plates Sp and 1'''''. **D)** Ink drawing to interpret the SEM images. Scale bar: 10 μ m.

In this work, we used the nomenclatural system used by [Balech \(1995\)](#) for genus *Alexandrium* to name sulcal plates of *Gambierdiscus*. We identified the sulcal plates by their relative position to other easily identified plates, taking into account that due to the torsion of the sulcal area all sulcal plates are

rotated almost 90° clockwise in relation to the equatorial plane. We identified Sa as the plate in contact to 6'', 1' and 1'' as [Taylor \(1979\)](#) did. This plate was named Smp by [Adachi and Fukuyo \(1979\)](#) and Sda by [Litaker et al. \(2009\)](#). Following the Balech's criteria for *Alexandrium*, we considered plate Sda

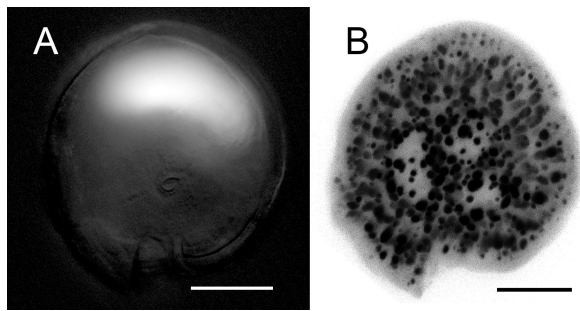


Figure 10. *Gambierdiscus silvae* (strain VGO1180). **A)** Epifluorescence LM image of nucleus stained with SYBR Green in relation to the whole cell, **B)** CLSM image of chloroplasts. Scale bars 20 μm .

to be the transition plate named t by Adachi and Fukuyo (1979), Taylor (1979), Litaker et al. (2009) and Nishimura et al. (2014). We agree with Litaker et al. (2009) and Nishimura et al. (2014) in the identification of the plate Sdp which was identified as a 7''' by Taylor (1979). Our interpretation of the sulcal plates disagrees with the names given by Litaker et al. (2009) in their Figure 33. Taking into account that it is optically reversed, we consider that their plates C1, Sda, Sm and t, are C6, 6'', Sa and Sda respectively under our nomenclature system. We considered that the plate named 2''' by other authors (Chinain et al. 1999; Faust 1995; Litaker et al. 2009; Xu et al. 2014) is plate Sp as did Adachi and Fukuyo (1979), Besada et al. (1982), Fraga et al. (2011) and Nishimura et al. (2014).

Although the shapes of some sulcal plates are important to differentiate species of a single genus, such as e.g. *Alexandrium* (Balech 1995), the observation of these plates in *Gambierdiscus* is very difficult because they are not flat, and as a consequence there are very few sulcal descriptions in the literature to be used to differentiate species

of this genus today. The presence of tall cells in cultures where coupling cells were observed suggests that these tall cells could be planozygotes. Morphological differences in plate shapes of different life cycle stages have been reported in another genus of Gonyaulacales, *Fragilidium* Balech, where in vegetative cells of *F. subglobosum* (von Stosch) Loeblich III, and *F. cf. duplocampaneforme* Nézan et Chomérat, Plate 1' does not contact Po, while in gametes, these two plates are in contact (Amorim et al. 2013). In the case of *Gambierdiscus* the zygotes could have a larger volume than vegetative cells incrementing the height of precingular and postcingular plates while keeping the same shape and size of apical and antapical plates (this study).

The cells of *G. australes* from Tenerife are in accordance with the original description from the Pacific Ocean (Chinain et al. 1999) both in morphology and dimensions.

Distribution

Gambierdiscus excentricus, was previously known from the area which is its type locality (Fraga et al. 2011). This species was found together with *G. australes* previously not reported in the Atlantic Ocean (Litaker et al. 2010; Parsons et al. 2012) along with the new species *G. silvae*. It is our opinion that *G. silvae* is probably the same species reported by Silva (1956) from the Cabo Verde archipelago. The Canary Islands are not unlike other regions having more than 2 species of *Gambierdiscus* as observed in French Polynesia (Chinain et al. 1999), Belize, Puerto Rico (Litaker et al. 2010), Japan (Nishimura et al. 2013) and Kiribati (Xu et al. 2014). Places with two species are very common (Litaker et al. 2010). It is interesting to note that the three species found in the Canary Islands have three different biogeographies. *G. excentricus* was only observed

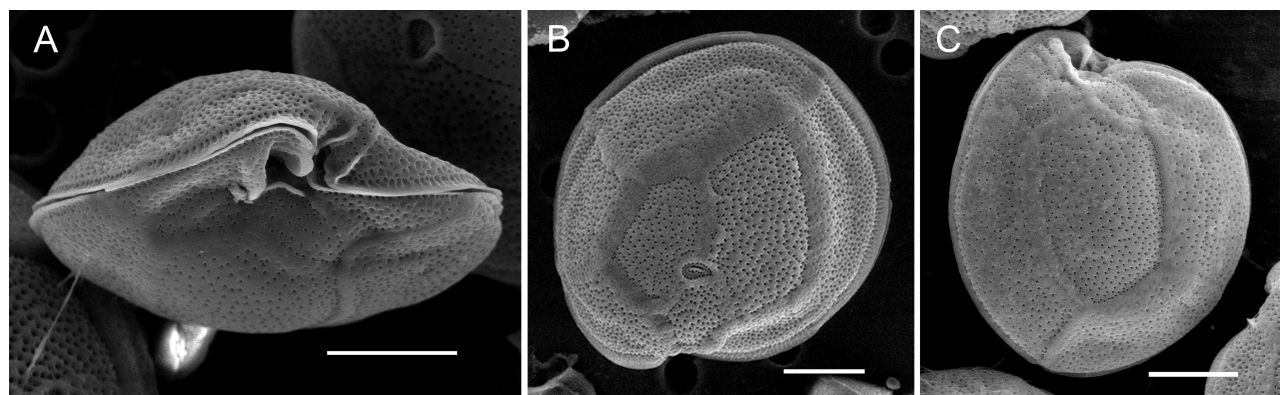


Figure 11. *Gambierdiscus australes* (strain VGO1161). SEM images **A)** Ventral view, **B)** Apical view, **C)** Antapical view. All scale bars: 20 μm .

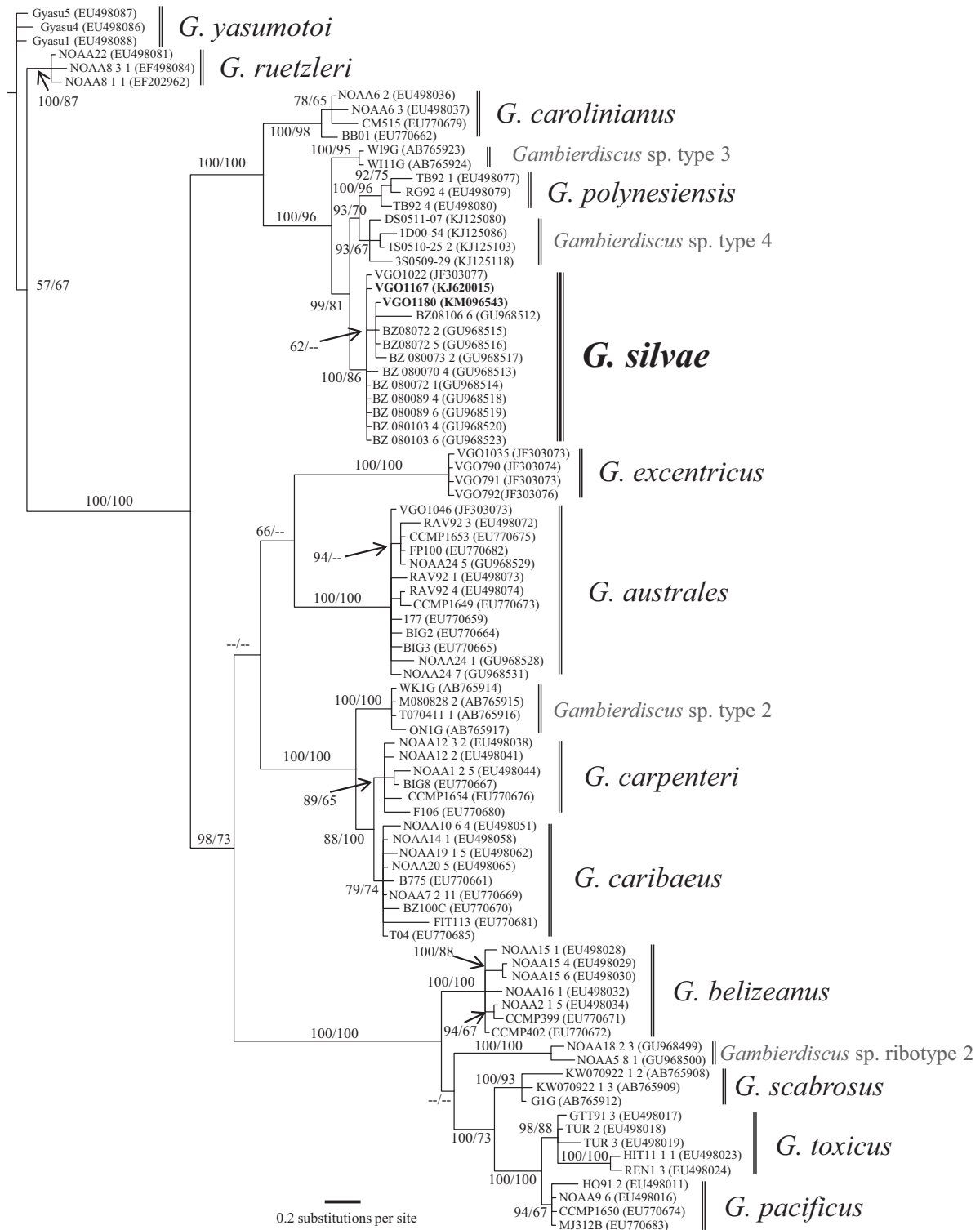
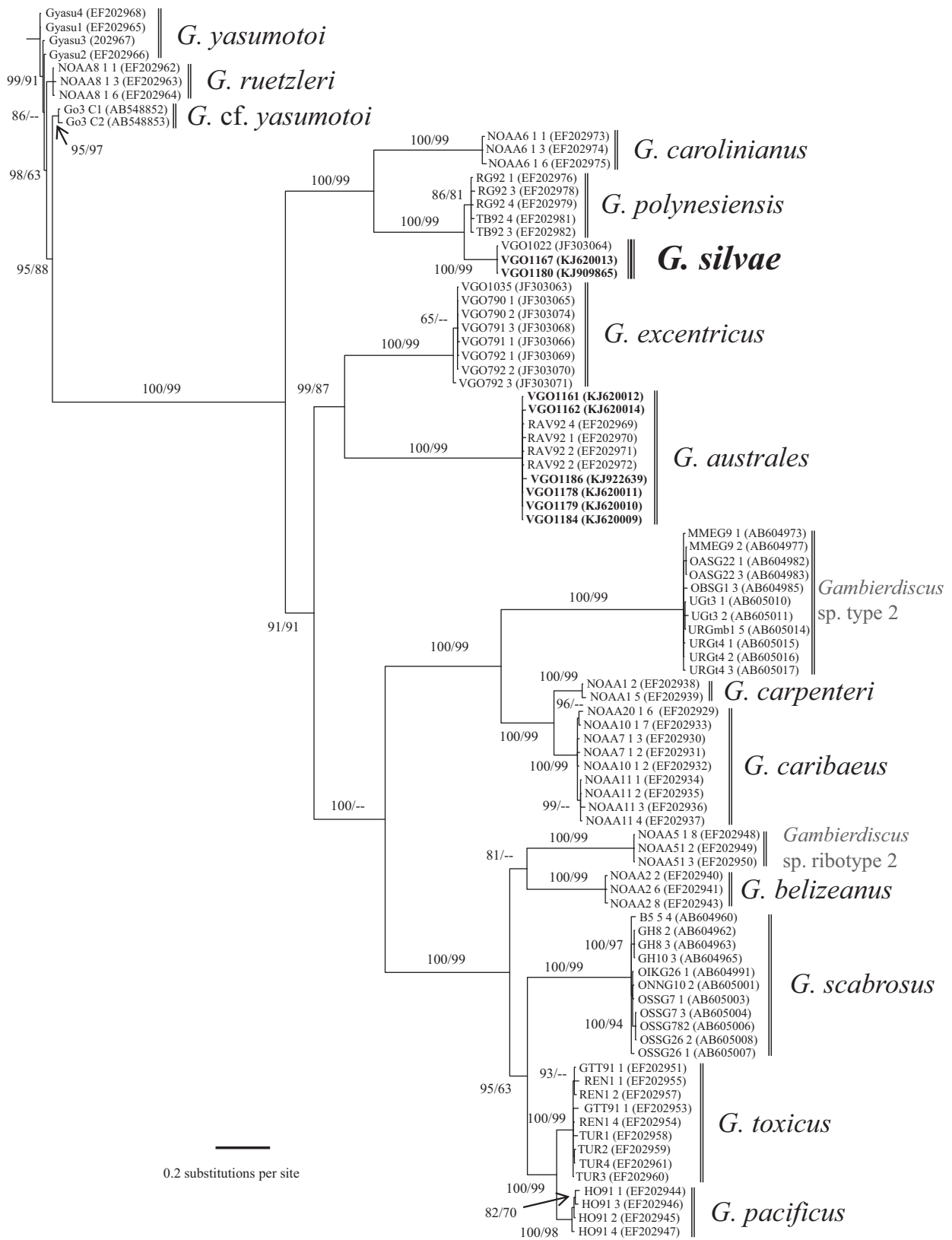


Figure 12. LSUrRNA phylogeny (D8–D10 region) showing the relationships between *Gambierdiscus silvae* and other *Gambierdiscus* species/phylotypes. New sequences from this study are in bold. Strain names and GenBank Acc.Nos. are detailed in each case. Internal nodes supports are posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens indicate bootstrap values <60.



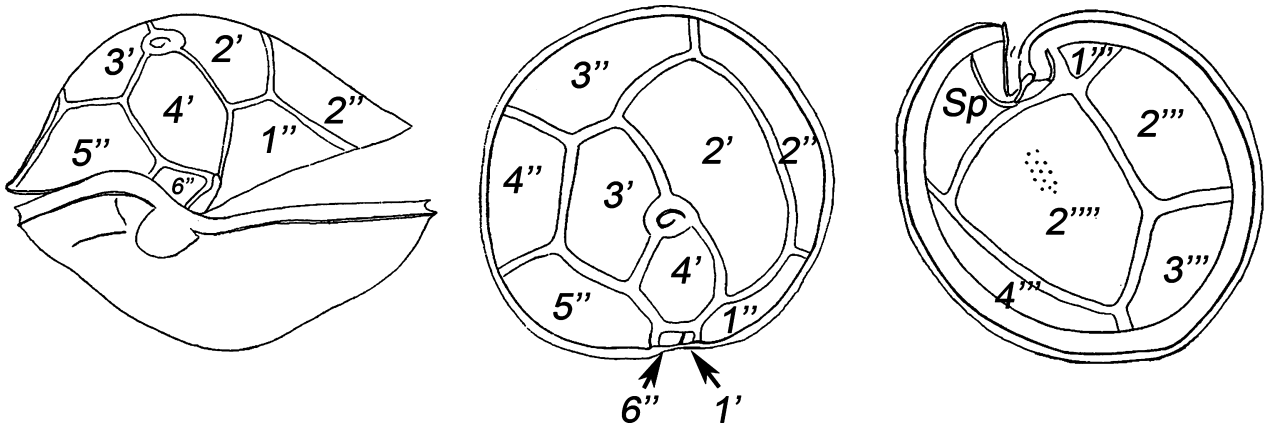


Figure 14. *Goniodoma* sp. modified after [Silva \(1956\)](#) with plates numbers added according to the criteria followed in this paper.

in temperate areas of the Atlantic Ocean, namely the Canary Islands ([Fraga et al. 2011](#)), the Moroccan coast (B. Ennaffah pers. comm.), and Brazil ([Nascimento et al. 2012](#)), but not in the Caribbean where other five *Gambierdiscus* species and two ribotypes were reported ([Litaker et al. 2010](#)). *G. australes* was reported from the Pacific Ocean, in French Polynesia, Hawaii and Japan, and from the Atlantic Ocean, in the Canary Islands. However it was absent from the Caribbean Sea. *G. silvae* was only observed in the Atlantic Ocean. In addition to the Canary Islands, it was reported from the Caribbean Sea as Ribotype 1 ([Litaker et al. 2010](#)). *Gambierdiscus* sp. type 4 ([Xu et al. 2014](#)) from the Pacific Ocean is both genetically and morphological very close to *G. silvae*. Nonetheless, *G. silvae* is closely related with *G. polynesiensis*, *G. sp* type 3 and *G. sp* type 4 ([Xu et al. 2014](#)) and these could represent recently diverged species undergoing an allopatric process of speciation after the closing of the Isthmus of Panama about 3 Ma as observed in *Ostreopsis*, another warm water benthic dinoflagellate ([Penna et al. 2010](#)).

The coexistence of congeneric species with apparently the same ecological niche is an outstanding topic that has attracted the attention of ecologists ([Levine and HilleRisLambers 2009](#)). The Canary Islands coastal waters have low variations in seasonal temperature suggesting an overall stable environment but tide pools are where environmental changes can be rapid and

large. [Hutchinson's \(1961\)](#) plankton paradox can be explained in this highly variable environment. Here the time for environmental changes is much shorter than the generation time of the cells, and hence, there is no competition among cells for the resources. In parallel to *Gambierdiscus*, several species of *Coolia* and *Prorocentrum* were also found in the same samples. It appears that there is no competition for resources among congeneric species and that the neutral theory of biodiversity proposed by [Hubbell \(2001\)](#) could be applied.

Phylogeny

The phylogenetic relationships (LSUrRNA) between *Gambierdiscus* species and phylotypes in our study were similar to those reported by other authors, including also our research group ([Fraga et al. 2011](#); [Litaker et al. 2010](#); [Nishimura et al. 2014](#); [Xu et al. 2014](#)). In the last years, several new genetic clades have emerged and could represent new undescribed *Gambierdiscus* species, pending a formal description. Before the present study, eight of these genetic groups, termed as *Gambierdiscus* sp. ribotypes 1 and 2 ([Litaker et al. 2009](#)) and six *G. sp.* types (types 1 to 6 following [Nishimura et al. \(2013\)](#) and [Xu et al. \(2014\)](#)) were found in the literature. Regarding *G. silvae* (previously *G. sp.* ribotype 1), the first sequences were reported by [Litaker et al. \(2010\)](#) from isolates in Belize (Caribbean Sea) and there

Figure 13. LSUrRNA phylogeny (D1–D3 region) of genus *Gambierdiscus*, including *Gambierdiscus silvae* and *G. australes* from Canary islands and other *Gambierdiscus* species and phylotypes. New sequences from this study are in bold. Strain names and corresponding GenBank Acc.Nos. are detailed in each case. Internal nodes supports are posterior probabilities (Bayesian analyses) and bootstrap values (Maximum Likelihood). Hyphens indicate bootstrap values <60.

existed a single cultivated strain (VGO1022) as reported by Fraga et al. (2011). The genetic relationships for *G. silvae* were already shown by Litaker et al. (2009), Nishimura et al. (2013) and Xu et al. (2014), but referring instead to *G. sp. ribotype 1*. The genetic data presented in our study includes two new strains (VGO1167 and VGO1180) of *G. silvae* (whose D1-D3 and D8-D10 LSUrRNA sequences resulted almost identical to the first isolate), together with sequences of four isolates of *G. australes*. Recently, Xu et al. (2014) reported a new phylotype (*Gambierdiscus* sp. type 4) in an intensive sampling in Kiribati Islands (Pacific), close to *G. silvae* and these authors indicated that it may represent a Pacific clade of *Gambierdiscus* sp. Ribotype 1. Nevertheless, there exist several accepted *Gambierdiscus* species which exhibit low levels of genetic differentiation in the LSUrRNA gene marker regions (Litaker et al. 2009). In this study, we used the average net genetic distances to determine the differences between groups of taxa (species or molecular clades), and to determine if *G. silvae* is separated from its sister clades to the same extent as other *Gambierdiscus* species. The genetic distances for close *Gambierdiscus* species calculated in our study were as follows: *G. yasumotoi*-*G. ruetzleri* (0.005 and 0.009, D8-D10 and D1-D3 respectively), *G. carpenteri*-*G. caribaeus* (0.005 and 0.052) and *G. toxicus*-*G. pacificus* (0.008 and 0.040). These values were lower or similar to those obtained between *G. silvae* and *G. polynesiensis* (0.012 and 0.047, D8-D10 and D1-D3 respectively), *G. silvae*-*G. sp. type 3* (0.015) and *G. silvae*-*G. sp. type 4* (0.010) in the D8-D10 alignment. After these results, it appears that the closest relative of *G. silvae* is *Gambierdiscus* sp. type 4 and that their genetic distance is comparable to that between *G. toxicus* and *G. pacificus*. Even LSUrRNA demonstrates a good resolution between *Gambierdiscus* species and genetic clades until now, but new studies continue adding complexity to the specific diversity in this genus. It would be advisable to explore other molecular markers and expand current LSUrRNA trees to others based on ITS or mitochondrial genes, as it has been extensively examined to better delineate species in other dinoflagellate genera (Penna et al. 2008, 2010).

Gambierdiscus silvae sp. nov. S. Fraga et F. Rodríguez

Description: Cells anterioposteriorly compressed, lenticular in shape with an epitheca slightly higher than hypotheca with averaged length $46 \pm 5 \mu\text{m}$,

depth $69 \pm 8 \mu\text{m}$, and width $64 \pm 9 \mu\text{m}$. It is round in apical view and has a thick smooth theca in apical and antapical plates, and slightly ornamented in pre- and postcingular plates, with many scattered pores. Plate formula is Po, 4', 0a, 6'', 6c, 6s?, 5''', 0p, 2'''''. Apical pore complex, Po is oval, has a fishhook-shaped slit, is centred in the epitheca and only slightly ventrally displaced. Plate 2' is hatchet-shaped and is the biggest of the apical plates. Plate 2'''' is the biggest of hypotheca and is very wide in relation to the length. The nucleus is U shaped and is located in the dorsal part of the cell with points towards the ventral side of the cell. Cells are photosynthetic having numerous small and round chloroplasts.

Etymology: This species is dedicated to the late Dr. Estela Sousa e Silva, a Portuguese researcher who was the first to report a *Gambierdiscus* species from samples taken in Cabo Verde in 1948 although as *Goniodoma* sp.

Holotype: Figure 2A; SEM-stub (designation CEDiT2014H47) deposited at the Senckenberg Research Institute and Natural History Museum, Centre of Excellence for Dinophyte Taxonomy, Germany.

The clonal strain VGO1022 has been deposited at Banco Español de Algas (BEA) Spain, under the strain name BEA 1164B. Strain VGO1022 barcoded in GenBank accession JF303064 and JF303077. Clone VGO1022 was collected on February 18th, 2010 as an epiphyte on small filamentous macroalgae and turf on a tidal pond in La Puntilla, Las Palmas, Canary Islands (Fig. 1).

Isotype: Formalin-fixed sample of strain VGO1022 (designation CEDiT2014I48) deposited at the Senckenberg Research Institute and Natural History Museum, Centre of Excellence for Dinophyte Taxonomy, Germany.

Type locality: La Puntilla, (28° 8.89' N, 15° 25.95' W) Las Palmas, Canary Islands, NE Atlantic Ocean.

Habitat and distribution: Marine, associated as epiphyte to seaweeds in a rocky tidal pool. It was observed in two of the Canary Islands, Tenerife and Gran Canaria. It is probably the same species that Silva (1956) reported from near Boavista Island, Cabo Verde, another Macaronesian archipelago. Based on the D8–D10 LSU sequence it was reported as Ribotype 1 from Belize (Litaker et al. 2010).

Methods

Source of specimens and culture conditions: Samples of diverse macroalgae were collected in tide pools at La Puntilla

(28° 8.9' N, 15° 26'W), in Las Palmas, Canary Islands in February 2010, and in Punta Hidalgo (28° 34'N, 16° 19'W), a rocky shore on the north coast of Tenerife, Canary Islands, in September 2013. The area was sampled only during low tide and samples were taken from high level tide pools to upper infralittoral zone accessible by simple snorkelling at no more than one meter depth. Samples of small mixed seaweeds were collected from tidal pools on the rocks during low tide, placed in plastic bottles and shaken. Afterwards, the gross materials were removed through a sieve and the remaining seawater was used for cell isolation. Isolation was carried out by a capillary pipette with the aid of a Zeiss Invertoscop D microscope (Carl Zeiss AG, Germany) and isolated cells were incubated in 96 microwell plates in full strength K/2 medium (Guillard and Hargraves 1993; Keller et al. 1987) made with seawater from Ría de Vigo with a salinity adjusted to 34 and incubated at 24 °C and a photon irradiance of about 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of PAR measured with a QSL-100 irradiator (Biospherical Instruments Inc. San Diego, CA, USA), at a 14:10 L:D photoperiod. The cultures are deposited at the Culture Collection of Microalgae (CCVIEO) of the Instituto Español de Oceanografía in Vigo. These and other cultures used in this study obtained from different sources are listed in Supplementary Material Table S1. As no significant differences were observed among wild and cultured cells, the study was based on cultured material.

Light microscopy: The cultured cells were observed alive or fixed with formalin. For plate pattern identification the cells were stained with Fluorescent Brightner 28 (Sigma, St Louis, MO, USA) following a modified Fritz and Triemer (1985) technique. Others were dissected, squashing the cells by pressing the cover slip over them and sometimes with the aid of sodium hypochlorite. The nuclei were stained using SYBR Green (Molecular Probes, Eugene, OR, USA) following the method of Figueroa and Bravo (2005). Light microscopy observations were carried out under a Leica DMLA light microscope (Leica Microsystems GmbH, Wetzlar, Germany) with phase contrast, differential interference contrast and epifluorescence. The photographs were taken with an Axiocam HRc (Carl Zeiss, Jena, Germany) digital camera. When the depth of field was not enough for the whole object, several pictures were taken at a series of different foci and automatically merged using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). The areas of each layer that were out of focus were erased and the layers flattened to get a final image with an enhanced depth of field. Cell sizes were measured with a Zeiss Axiovert 125 (Carl Zeiss, Jena, Germany) microscope with a Moticam 1 digital camera (Motic Spain, S.L.U.) and its software. Confocal laser scanning microscopy (CLSM) images were taken with a Leica TCS SP5 confocal microscope using calcofluor for staining of thecal plates and chlorophyll autofluorescence for observation of chloroplasts.

Sample preparations for SEM: Exponentially growing cultures (5 mL) were fixed with glutaraldehyde a final concentration of 2%. After two hours at room temperature, they were rinsed three times with distilled water and dehydrated in a series of 30, 50, 75, 95 and 100% EtOH followed by Hexamethyldisilazane. After being air dried overnight, they were coated with gold with a K550 X sputter coater (Emitech Ltd., Ashford, Kent, UK) and observed with a Phillips XL30 or a FEI Quanta 200 scanning electron microscopes (FEI Company, Hillsboro, OR, USA).

Nomenclature: In this study, the modified Kofoid (1909) tabulation nomenclature proposed by Besada et al. (1982) was used. This was the system used in the last two descriptions of new species of *Gambierdiscus*, *G. excentricus* (Fraga et al. 2011) and *G. scabrosus* (Nishimura et al. 2014), because it allows comparisons with other genera of Gonyaulacales based

on plates homologies. The terms “length” as apical/antapical distance, “width” as transdiameter and depth for dorso/ventral distance were used for the dimensions.

DNA extraction: Exponentially growing cultures of *Gambierdiscus* (1 mL) were harvested by centrifugation (13.000 rpm, 2 min) using an Eppendorf 5424R centrifuge (Eppendorf AG, New York, USA), the cellular pellets were rinsed in 1 mL distilled water, centrifuged again and the supernatant discarded. Samples were frozen overnight at -20 °C and DNA extraction was done using a modified Chelex procedure (Richlen and Barber 2005). 100 μL of 10% Chelex 100 (Bio-Rad, Hercules, CA, USA) in dH_2O was added and samples were transferred to 200 μL tubes. The samples were heated to 95 °C in a Surecycler 8800 thermocycler (Agilent Technologies, Santa Clara, CA, USA) for 10 min, then vortexed. The heating and vortex steps were done twice. Samples were centrifuged (13.000 rpm, 2 min) and the supernatants were transferred to clean 200 μL tubes avoiding to carryover the Chelex beads. Genomic DNA was quantified and checked for its purity in a Nanodrop Lite spectrophotometer (ThermoScientific, Waltham, MA, USA). When necessary, DNA samples were further purified using a standard ethanol precipitation protocol (Surzycki 2000) and stored at -20 °C until further processing.

PCR amplification and DNA sequencing: The D1–D3 and D8–D10 regions of the LSU rRNA gene were amplified using the pairs of primers D1R/LSUB (5'-ACCCGCTGAATTTAAGCATA-3'/5'-ACGAACGATTTGCACGTCAG-3') and FD8/RB (5'-GGATTGGCTCTGAGGGTTGGG-3'/5'-GATAGGAAGAGCC-GACATCGA-3') (Chinain et al. 1999; Litaker et al. 2003; Scholin et al. 1994) respectively. Amplification reaction mixtures (25 μL) contained 4 mM MgCl_2 , 0.5 pmol of each primer, 0.8 mM of dNTPs, 0.25 units Taq DNA polymerase (Qiagen, CA, USA), and 1–2 μL from the Chelex extractions. The DNA was amplified in a Surecycler 8800 thermocycler following the conditions detailed elsewhere (Chinain et al. 1999; Litaker et al. 2003). A 10 μL aliquot of each PCR reaction was checked by agarose gel electrophoresis (1% TAE, 50 V) and GelRed™ nucleic acid gel staining (Biotium, Hayward, CA, USA).

The PCR products were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Purified DNA was sequenced using the Big Dye Terminator v3.1 Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and migrated in an AB 3130 sequencer (Applied Biosystems) at the CACTI sequencing facilities (Universidad de Vigo, Spain). The D1–D3 and D8–D10 sequences obtained in this study were deposited in GenBank (for Acc.Nos. see Supplementary Material Table S1 and Figures 12–13).

Phylogenetic analyses: LSU sequences were inspected and aligned using CLUSTALW multiple alignment in Geneious® Pro 5.6.6 (Biomatters Ltd.). Net average genetic distances ($d_A = d_{XY} - (d_X + d_Y)/2$, where d_{XY} is the average distance between groups X and Y, and d_X and d_Y are the mean within-group distances (Nei 1987), were calculated between *Gambierdiscus* clades for the original alignments using MEGA 6 software. D1–D3 and D8–10 alignments included 1064 and 800 positions. In the case of D8–D10 alignment, poorly aligned positions and divergent regions were checked using GBLOCKS (Castresana 2000). A final number of 581 bases (72% of the original 800 positions) were saved by GBLOCKS and used in further genetic analyses. Phylogenetic model selection (ML) was performed on MEGA 6. A K2+G model was selected for the D1–D3 and D8–D10 alignments, with gamma shape parameter = 1.02 and 0.74 respectively. D8–D10 *Gambierdiscus yasumotoi* sequences were used to root the trees. The phylogenetic relationships were also determined using

Bayesian phylogenetic inference and in this case the substitution models were obtained by sampling across the entire general time reversible (GTR) model space following the procedure described in Mr. Bayes v3.2 manual. Bayesian trees were performed with MrBayes v3.2 (Huelsenbeck and Ronquist 2001) and the program parameters were `statefreqpr=dirichlet(1,1,1,1)`, `nst=mixed`, `rates=gamma`. The phylogenetic analyses involved two parallel analyses, each with four chains. Starting trees for each chain were selected randomly using the default values for the MrBayes program. The corresponding number of unique site patterns was 772 and 210 in D1–D3 and D8–D10 analyses. The number of generations used in these analyses was 200,000. Posterior probabilities were calculated from every 100th tree sampled after log-likelihood stabilization (“burn-in” phase). All final split frequencies were < 0.02 . Maximum Likelihood phylogenetic analyses were conducted in MEGA 6 (for D1–D3 alignment) and PhyML 3.0 (Guindon et al. 2010) for D8–D10 sequences on the South of France bioinformatics platform (<http://www.atgc-montpellier.fr/phyml>). Bootstrap values were estimated from 1000 replicates. Overall topologies by ML and Bayesian inference method were very similar. The phylogenetic tree was represented using the Bayesian inference with posterior probability and bootstrap values from the ML method.

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.protis.2014.09.003>.

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