

RESEARCH NOTE

Circumtropical distribution of the epiphytic dinoflagellate *Coolia malayensis* (Dinophyceae): Morphology and molecular phylogeny from Puerto Rico and Brazil

Fernando Gómez,^{1*} Dajun Qiu,² Ernesto Otero-Morales,³ Rubens M Lopes¹ and Senjie Lin⁴

¹Laboratory of Plankton Systems, Department of Biological Oceanography, Oceanographic Institute, University of São Paulo, São Paulo, Brazil, ²CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Science, Guangzhou, China, ³Department of Marine Sciences, University of Puerto Rico, Mayagüez, Puerto Rico and ⁴Department of Marine Sciences, University of Connecticut, Groton, Connecticut, USA

SUMMARY

The dinoflagellate genus *Coolia*, which contains potentially toxic species, is an important component of epiphytic assemblages in marine ecosystems. The morphology of *C. malayensis* has been illustrated from strains isolated in Asia and Oceania. In this study, strains of *C. malayensis* isolated from the Caribbean Sea in Puerto Rico, and for the first time from the South Atlantic Ocean in Brazil, were investigated by light, epifluorescence and scanning electron microscopies. No significant morphological differences between these new strains and other geographically distant strains of *C. malayensis* were observed. In the LSU rDNA phylogeny, the *C. malayensis* sequences from Brazil and Puerto Rico branched within the clade of strains from Oceania and Asia. The recently described species *C. santacroce* branched as a sister group of *C. monotis*, and *C. palmyrensis* was basal to the combined group of *C. monotis/C. malayensis/C. santacroce*. A tentative undescribed species from Florida and New Zealand branched as a sister group of *C. malayensis*. Our results confirm that *C. malayensis* showed a cosmopolitan distribution in tropical to subtropical waters, while the type species *C. monotis* remains endemic for the Mediterranean Sea and the temperate North Atlantic.

Key words: benthic dinophyta, Caribbean Sea, epiphyte, harmful algal blooms, microalgae, South Atlantic Ocean, toxic dinoflagellata.

The species of the genus *Coolia* Meunier are an important component of benthic dinoflagellate assemblages, especially in tropical and subtropical waters (Karafas *et al.* 2015; Wakeman *et al.* 2015). The type species, *C. monotis* Meunier, was described in the temperate waters and other species were described in tropical waters: *C. tropicalis* M. A. Faust, *C. areolata* Ten-Hage, J. Turquet, J. P. Quod & Couté and *C. canariensis* S. Fraga (Meunier 1919; Faust 1995; Ten-Hage *et al.* 2000; Fraga *et al.* 2008). Further studies reported a considerable morphological variability in *C. monotis*, and the molecular phylogenetic analyses revealed genetic divergences among *C. monotis* isolates from the European Atlantic and Mediterranean coasts, and those from other world regions (Penna *et al.* 2005; Dolapsakis *et al.* 2006). The clade containing a sequence from the Netherlands was attributed to the type species, *C. monotis*. A sister group of sequences from

Eastern Asia, Oceania and the Caribbean Sea were attributed to the new species *C. malayensis* Leaw, P.-T. Lim & Usup (Leaw *et al.* 2010; Mohammad-Noor *et al.* 2013; Momigliano *et al.* 2013; David *et al.* 2014; Rhodes *et al.* 2014; Tawong *et al.* 2015). Hoppenrath *et al.* (2014) considered that *C. malayensis* is a synonym of *C. monotis*, while Karafas *et al.* (2015) described two new species within the *C. monotis/C. malayensis* clade: *C. santacroce* S. Karafas, C. Tomas & R. York and *C. palmyrensis* S. Karafas, C. Tomas & R. York. Karafas *et al.* (2015) reported the presence of *C. monotis* for the first time in the coasts of the Northwestern Atlantic Ocean.

The species diagnosis within the genus *Coolia* was traditionally based on the epithecal tabulation. The width/length ratio of plate 7'' is approximately 1 for *C. monotis*, 2 for *C. canariensis* and *C. areolata*, and 4 for *C. tropicalis*. In contrast, *Coolia monotis* and *C. malayensis* were separated based on the hypothecal tabulation. In *C. monotis*, the third (3'') and fourth (4'') postcingular plates are almost equal in size, while in *C. malayensis* the plate 3''' is the largest hypothecal plate (Leaw *et al.* 2010). However, Jeong *et al.* (2012) found cells whose 3''' plate size was similar to the 4''' plate size. Besides, like the European strains of *C. monotis*, the shape of 3' plate of most cells of the Korean strains of *C. malayensis* is pentagonal, while that of the Malaysian strains is quadrangular (Leaw *et al.* 2010). The recently described species, *C. palmyrensis* and *C. santacroce*, have been separated from *C. monotis* and *C. malayensis* based on the cell size, apical pore size, and the size and density of pores. These new species have fewer pores than *C. monotis* and *C. malayensis* (Karafas *et al.* 2015).

In this study, we analyzed morphology and molecular data of strains isolated from Puerto Rico and Brazil. The latter strain represents the first study of the genus *Coolia* in the South Atlantic Ocean. The present study provides data needed for better understanding on the distribution of

*Present address: Carmen Campos 3, E-11500 Puerto Santa María, Spain.

Email: fernando.gomez@fitoplancton.com

Communicating editor: Mitsunobu Kamiya

Received 9 November 2015; accepted 15 April 2016.

C. monotis/C. malayensis in the world ocean and on the variations in the morphology and DNA sequences.

We obtained morphological characteristics using light and electron microscopies for the Puerto Rico (Fig. S1 in the Supporting Information) and Brazilian (Figs 1, S2 in the Supporting Information) clonal cultures. The isolation, culturing, morphological and molecular methods are described in the Appendix S1 in the Supporting Information. The cells from both locations were obliquely roundish in lateral view and contained many golden-brown chloroplasts. The anterior-posterior axis was characteristically oblique; the apex is displaced dorsally and the antapex is displaced ventrally. The hypotheca was slightly larger than the epitheca. Cells in clonal culture

ranged from 22 to 33 μm long, and 19 to 33 μm wide. In apical view, the apical pore plate (Po) was dorsally displaced and located among the second (2'), third (3') and first (1') apical plates (Figs 1A,H-I,R,W, S1b, n-p, S2). The Po was slightly curved, measuring around 6–9 μm (Figs 1H-I,X, S1o-p). Plate 1' was oblong, hexagonal situated on the left side of the epitheca in apical view with its right side located in the middle of the dorso-ventral part of the cell, touching plate 6'', which was the largest plate of the epitheca (Figs 1A-D,M-N, R,T-W, S1m-o, S2). Plate 2' was the smaller in the apical series, and harbored the Po in most of its area. It was adjacent to the plates 2'', 3'', 4'' and 3' (Figs 2Q-R,V-W, S1l, n-o, S2). Plate 3' was pentagonal and situated between plates

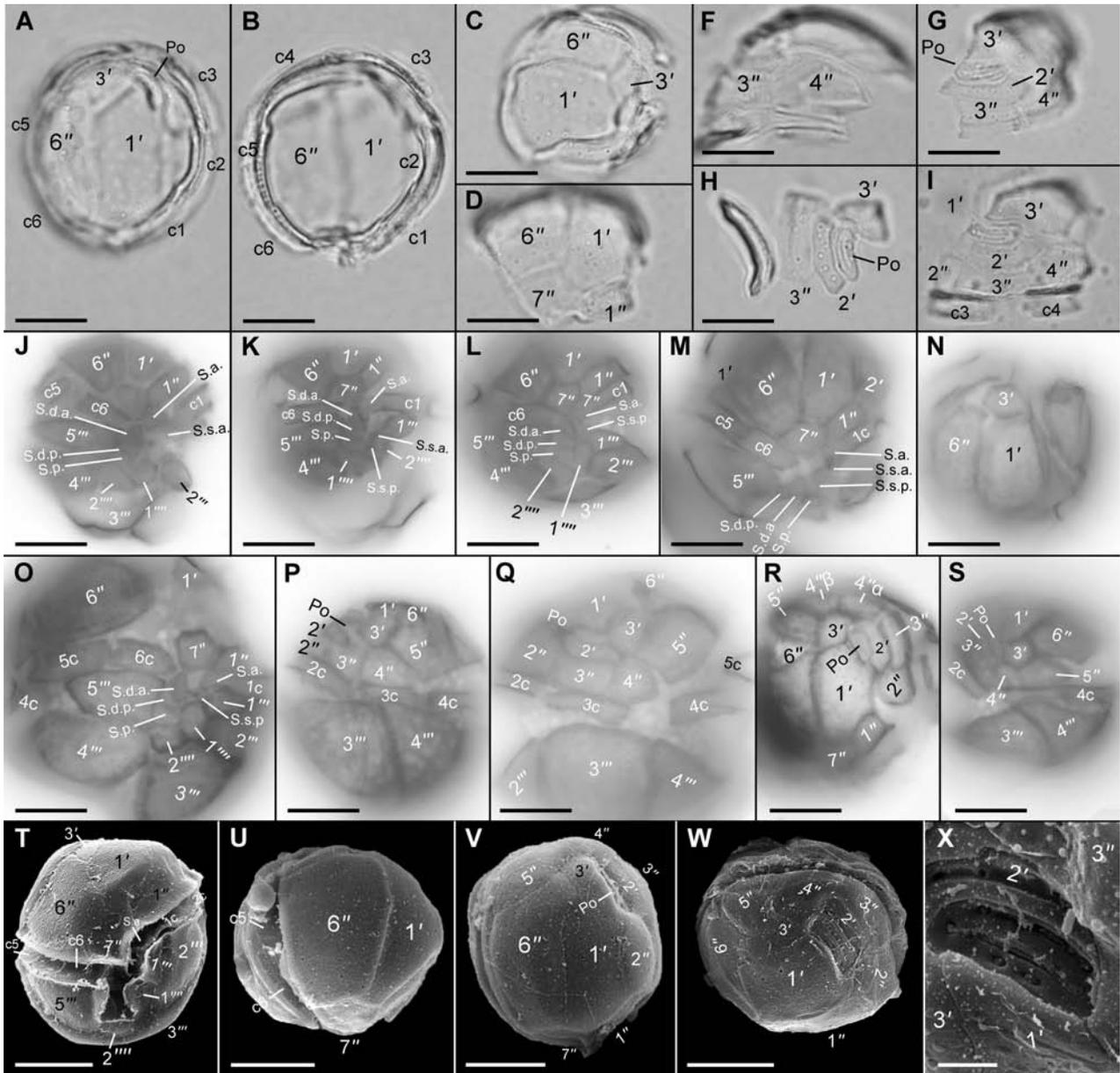


Fig. 1. Micrographs of the Brazilian strain of *Coolia malayensis*. (A–I) Light micrographs, (A, B) detail of cingular plates, (C–I) detail of the epithecal plates, (J–S) epifluorescence microscopy stained with Fluorescent Brightener, (T–X) scanning electron micrographs, (T) ventral view, (U–V) apical view, (W) dorsal-apical view, (X) detail of the apical pore plate. Scale bars = 10 μm , except 2 μm in X.

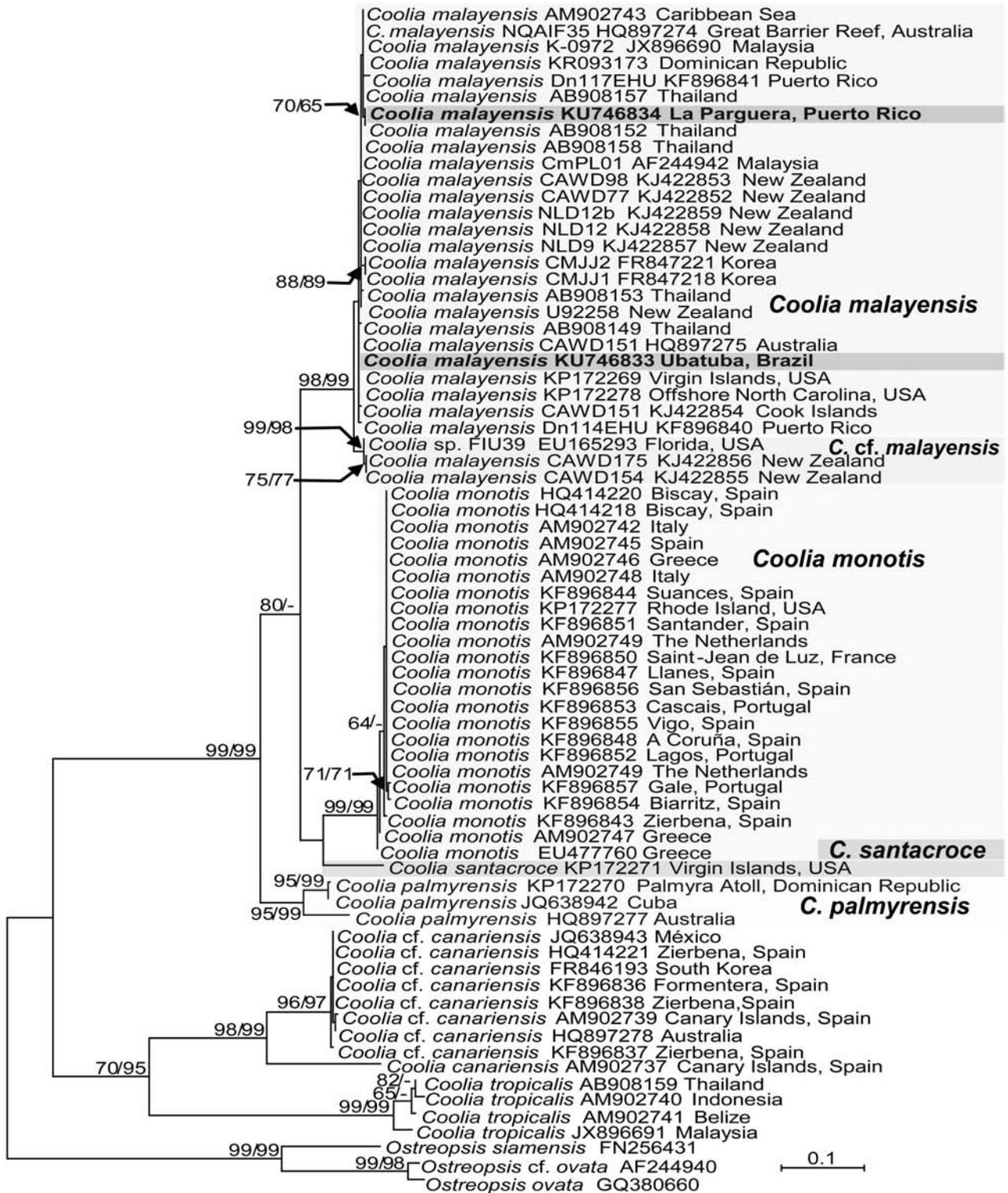


Fig. 2. Maximum likelihood tree of *Coolia* spp. based on the LSU rDNA (D1–D2) sequences. Sequences obtained in this study are bold-typed. Support of nodes is based on bootstrap values of Maximum Likelihood (ML)/Neighbor Joining (NJ) methods with 1000 resamplings. Only values greater than 60 are shown. *Ostreopsis* spp. were used as an outgroup.

1', Po, 2', 4'', 5'' and 6'' (Figs 1H–I, Q–R, S1k–o, S2). Plate 1'' was the smallest of the precingular series and connected plates 7'', 1' and 2''. (Figs 1D, J–M, R, T, S1e–f, s, S2). Plate 2''

was pentagonal, larger than 1''. The rectangular plate 3'' was situated on the dorsal side of the epitheca, adjacent and adjacent to 1'', 3'', 2' and 1' (Figs 2Q–R, T, V–W, S1n–o, 2s to 2'',

2' and 4" (Figs 1P–R,W, S1a–c,k–l,o, S2). Plate 4" touched 3", 2', 3' and 5". In some cells of the Brazilian strain, plate 4" appeared to be divided into two plates (Fig. 1R). Plate 5" was quadrangular, and surrounded by plates 4", 3' and 6" (Figs 1P–Q,W, S1a,k,o). The pentagonal plate 6" was the largest precingular plate and occupied the majority of the epitheca on the left side of the cell (Fig. 1M,U–V, S1m–n, S2). It was surrounded by plates 7", 1', 3' and 5". Plate 7" was pentagonal, about as wide as long, and surrounded by plates 6", 1' and 1" (Figs 1D,L–M, S1e–f,m–n, S2). The cingulum was equatorial with six cingular plates (Figs 1A–B,M, P–Q, S1d). The sulcus was narrow and did not reach the antapex of the cell. Sulcal plates were very hard to distinguish, since the sulcus was deeply excavated and indented, but it was still possible to distinguish the posterior sulcal plate (S.p.), located just above plate 2"" in ventral view (Figs 1J–M, O, S1e). The hypotheca contained seven plates: five postcingular (5"" and two antapical plates (2""). Plates 1"" and 5"" were small and triangular. Plates 3"" and 4"" were equal in size and the largest plates found on the hypotheca. Plate 3"" was quadrangular and in a mid-dorsal position of the hypotheca (Figs 1O–Q,S, S1g–l). The antapical plate 1"" was small and located adjacent to 1"", 2"" and 3"". Plate 2"" was very small and adjacent to plates 3"", 4"", 5"" and 1"" (Figs 1O, S1g–h). Plates 3"", 4"" and 5"" surrounded the smallest antapical plate 2"", which was just below and slightly to the right of plate 1"". The plate formula was Po, 3', 7", 6c, 5 + s, 5"", 2"" or alternatively interpreted as Po, 4', 6", 6c, 5 + s, 5"", 2"" (Figs 1, S2, S4g–h).

Two new LSU rDNA sequences were obtained from the strains isolated from Brazil and Puerto Rico. In the LSU rDNA molecular phylogeny the new sequences of *C. malayensis* from Brazil and Puerto Rico branched in a clade with sequences of strains from Asia, Oceania, the Caribbean Sea and one sequence from temperate Atlantic waters of North America (Fig. 2). The strains *Coolia* sp. FIU39 from Florida and *C. malayensis* CAWD154 and CAWD175 from New Zealand branched in a sister clade of the main clade of *C. malayensis* (Fig. 2). Further studies are necessary to confirm whether these strains should be considered as a new species or just distinct genotypes. The strains of *C. monotis* from Europe and one from the Atlantic coasts of North America formed a clade distinct from *C. malayensis*. The species *C. santacroce* branched in a sister group with *C. monotis*. The clade of *C. palmyrensis*, with sequences from Australia and the Caribbean Sea, was basal to the combined clade of *C. monotis/malayensis/santacroce* (Fig. 2). The strains of *C. canariensis* branched into two distinct groups, suggestive of existence of cryptic species.

Our sequencing effort also yielded two new ITS sequences of *C. malayensis* from the Brazilian and Puerto Rican strains. Currently, there are much fewer ITS sequences available in GenBank than the LSU rDNA. Our strains from Brazil and Puerto Rico differed in 25 bp and both branched in a clade with the strains from Asia and the Caribbean Sea. The sequences of a strain reported as CM01 from Malaysia showed a slight divergence from the main clade of *C. malayensis* (Fig. S3 in the Supporting Information).

Meunier (1919) described *C. monotis* from oyster beds in the North Sea at Nieuport, Belgium. He reported an anomalous tabulation with an epitheca composed of eight

precingular plates. In contrast, all further observations of the European *C. monotis* showed seven precingulars (Fig. S4b–c in the Supporting Information; Balech 1956; Penna *et al.* 2005; Dolapsakis *et al.* 2006; Aligizaki and Nikolaidis 2006; Laza-Martínez *et al.* 2011; David *et al.* 2014). Jeong *et al.* (2012) observed that under culture conditions some cells of *C. malayensis* showed the plate 4' split into two plates. The same phenomenon was observed in some specimens of the Brazilian strain (Fig. 1R). The occurrence of more than seven precingular plates is rare in gonyaulacoid dinoflagellates (except for genera with an exceptional multiplication of plates such as *Fragilidium* Balech ex A. R. Loeb. and *Pyrophacus* F. Stein). In addition, Jeong *et al.* (2012) found seven cingular plates, which is an exceptional feature for gonyaulacoid dinoflagellates. We should consider whether the culture conditions can induce the anomalous tabulations.

Meunier (1919) described *C. monotis* with an unusual tabulation, a very large plate 5' and a plate 3' with a hexagonal shape (Fig. S4a in the Supporting Information). There is no further information on the morphology of specimens collected from the type locality. Two sequences of *C. monotis* from the Netherlands, close to the type locality, branched with the other European Atlantic and Mediterranean strains (Penna *et al.* 2005). The tabulation of *C. monotis* by Meunier (1919) does not agree with further observations because subsequent studies in the European Atlantic coasts showed a pentagonal plate 3' and seven precingular plates (Balech 1956; Laza-Martínez *et al.* 2011). The situation seems to reappear in the case of *C. malayensis*, described as result of the split of *C. monotis*. The line drawings by Leaw *et al.* (2010) represented *C. malayensis* with a plate 3' with a quadrangular shape (Fig. S4d in the Supporting Information). However, all further studies showed plate 3' with a pentagonal shape, even the one examined the specimen collected nearby the type locality (Tawong *et al.* 2015, Fig. S4e–f).

The morphological distinction between *C. monotis* and *C. malayensis* is difficult. Some authors considered that they are synonyms (Hoppenrath *et al.* 2014), while others have proposed additional new species within the clade of *C. monotis* and *C. malayensis* (Karafas *et al.* 2015). The epitheca tabulation is commonly used for genus or species diagnosis. However, in the case of *C. malayensis* the hypothetical tabulation was the morphological basis to propose *C. malayensis* as an independent species from *C. monotis* (Leaw *et al.* 2010). Plate 3"" is the largest hypotheca in the original description of *C. malayensis*, whereas in *C. monotis*, plates 3"" and 4"" are almost equal in size (Leaw *et al.* 2010). Our results agrees with Jeong *et al.* (2012) that reported similar size in the plates 3"" and 4"" of *C. malayensis*.

For the new species *C. palmyrensis* and *C. santacroce*, the separation was based on the cell size, apical pore size, and the size and density of pores (Karafas *et al.* 2015). It is uncertain whether such characters justify the erection of new species. Karafas *et al.* (2015) did not solve the morphological distinction *C. monotis/C. malayensis*. This situation could reappear in the case of *C. canariensis*, which was phylogenetically divided into two clades, suggesting a cryptic speciation (Fig. 2; David *et al.* 2014).

Although *Coolia* is more commonly found in warm waters, curiously it was first described from temperate waters (Meunier 1919). This was very likely due to the paucity of

studies on epiphytic dinoflagellates in warm waters in the past. *Coolia* could have been reported in even earlier studies because one of the line drawings of *Glenodinium obliquum* C. H. G. Pouchet from the coast of Britany resembles *Coolia* (Pouchet 1883). It is usually assumed that benthic organisms have more dispersal constraints than their planktonic counterparts. For example, more than 50% of the benthic fauna is endemic in the Mediterranean Sea (Fredj *et al.* 1992). Up to date, *C. monotis* has been reported exclusively in the Atlantic and the Mediterranean Sea, while *C. malayensis* is mostly found in the Pacific and Caribbean Sea, with the only exception of a strain from North Carolina, USA, close to Rhode Island from where the strain of *C. monotis* were collected (Karafas *et al.* 2015). The phylogenetic placement of strains from Puerto Rico and Brazil indicates that the apparent geographic separation is subtle. Our results based on the LSU rDNA and ITS markers reveal that no significant differences exist between the populations in the South and North Atlantic Ocean, and Oceania and the Asian coasts (Fig. 2). Consequently, *C. malayensis* shows a circumtropical cosmopolitan distribution and low degree of endemism. This feature has been observed in other epiphytic toxic dinoflagellates (Gómez *et al.* 2015). In contrast, the distribution of the type species, *C. monotis*, was until recently restricted to the coasts of Europe and the Mediterranean Sea (Fig. S5 in the Supporting Information). The report of *C. monotis* in Rhode Island, USA (Karafas *et al.* 2015) has extended the range but still within the Atlantic Ocean. Until now, the populations of *C. monotis* and *C. malayensis* do not overlap geographically, and *C. monotis* shows a more cold water distribution. Taking into account the intense maritime traffic or exchange of live shellfish to and from Europe, we can expect that *C. monotis* will likely be introduced into other regions, as well as *C. malayensis* in the warm waters of the Mediterranean Sea. Both species are morphologically similar, and the existence of physiological differences between them remains an open question. It is clear that to fully understand the global distribution and population differentiation in the genus *Coolia*, further studies with broader sampling of different geographic strains are required. Such studies are also needed in order to more precisely resolve the taxonomic relationships of these taxa. Epiphytic dinoflagellates from numerous regions of the world's oceans remain unexplored and our knowledge of the biogeography and distribution of *Coolia* remains incomplete.

ACKNOWLEDGMENTS

This research is supported by the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (grant numbers BJT 370646/2013-14 to F. G., and 402759/2012-5 and 311936/2013-0 to R. M. L.), and United States National Science Foundation (EF-0629624 to S. L.).

REFERENCES

Aligizaki, K. and Nikolaidis, G. 2006. The presence of the potentially toxic genera *Ostreopsis* and *Coolia* (Dinophyceae) in the North Aegean Sea, Greece. *Harmful Algae* **5**: 717–30.

Balech, E. 1956. Études des dinoflagellés du sable de Roscoff. *Rev. Algol.* (n. ser.) **2**: 29–52.

David, H., Laza-Martínez, A., Miguel, I. and Orive, E. 2014. Broad distribution of *Coolia monotis* and restricted distribution of *Coolia* cf. *canariensis* (Dinophyceae) on the Atlantic coast of the Iberian Peninsula. *Phycologia* **53**: 342–52.

Dolapsakis, N. P., Kilpatrick, M. W., Economou-Amilli, A. and Triantafyllos, T. 2006. Morphology and rDNA phylogeny of a Mediterranean *Coolia monotis* (Dinophyceae) strain from Greece. *Sci. Mar.* **70**: 67–76.

Faust, M. A. 1995. Observation of sand-dwelling toxic dinoflagellate (Dinophyceae) from widely differing sites, including two new species. *J. Phycol.* **31**: 996–1003.

Fraga, S., Penna, A., Bianconi, I., Paz, B. and Zapata, M. 2008. *Coolia canariensis* sp. nov. (Dinophyceae), a new nontoxic epiphytic benthic dinoflagellate from the Canary Islands. *J. Phycol.* **44**: 1060–70.

Fredj, G., Bellan-Santini, D. and Meinard, M. 1992. État des connaissances sur la faune marine méditerranéenne. *Bull. Inst. Oceanogr. Monaco* **9**: 133–45.

Gómez, F., Qiu, D., Lopes, R. M. and Lin, S. 2015. *Fukuyoya paulensis* gen. et sp. nov., a new genus for the globular species of the dinoflagellate *Gambierdiscus* (Dinophyceae). *PLoS One* **10** (4): e0119676.

Hoppenrath, M., Murray, S. A., Chomérat, N. and Horiguchi, T. 2014. *Marine Benthic Dinoflagellates-Unveiling their World-wide Biodiversity*. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Jeong, H. J., Yih, W., Kang, N. S. *et al.* 2012. First report of the epiphytic benthic dinoflagellates *Coolia canariensis* and *Coolia malayensis* in the waters off Jeju Island, Korea: Morphology and rDNA sequences. *J. Eukaryot. Microbiol.* **59**: 114–33.

Karafas, S., York, R. and Tomas, C. 2015. Morphological and genetic analysis of the *Coolia monotis* species complex with the introduction of two new species, *Coolia santacroce* sp. nov. and *Coolia palmyrensis* sp. nov. (Dinophyceae). *Harmful Algae* **46**: 18–33.

Laza-Martínez, A., Orive, E. and Miguel, I. 2011. Morphological and genetic characterization of benthic dinoflagellates of the genera *Coolia*, *Ostreopsis* and *Prorocentrum* from the South-Eastern Bay of Biscay. *Eur. J. Phycol.* **46**: 46–65.

Leaw, C.-P., Lim, P.-T., Cheng, K.-W., Ng, B.-K. and Usup, G. 2010. Morphology and molecular characterization of a new species of thecate benthic dinoflagellate, *Coolia malayensis* sp. nov. (Dinophyceae). *J. Phycol.* **46**: 162–71.

Meunier, A. 1919. Microplankton de la mer Flamande. III Les Péridiniens. *Mem. Mus. R. Hist. Nat. Belgium* **8**: 3–116.

Mohammad-Noor, N., Moestrup, Ø., Lundholm, N. *et al.* 2013. Autecology and phylogeny of *Coolia tropicalis* and *Coolia malayensis* (Dinophyceae), with emphasis on taxonomy of *C. tropicalis* based on light microscopy, scanning electron microscopy and LSU rDNA. *J. Phycol.* **49**: 536–45.

Momigliano, P., Sparrow, L. and Heomann, K. 2013. The diversity of *Coolia* spp. (Dinophyceae Ostreopsidaceae) in the central Great Barrier reef region. *PLoS One* **8**(10): e79278.

Penna, A., Vila, M., Fraga, S. *et al.* 2005. Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8s rDNA sequences. *J. Phycol.* **41**: 212–5.

Rhodes, L., Smith, K., Giménez Papiol, G., Adamson, J., Harwood, T. and Munday, R. 2014. Epiphytic dinoflagellates in sub-tropical New Zealand, in particular the genus *Coolia* Meunier. *Harmful Algae* **34**: 36–41.

Pouchet, G. 1883. Contribution à l'histoire des cilio-flagellés. *J. Anat. Physiol. Norm. Pathol. Homme Anim.* **19**: 399–455.

Tawong, W., Nishimura, T., Sakanari, H., Sato, S., Yamaguchi, H. and Adachi, M. 2015. Characterization of *Gambierdiscus* and *Coolia* (Dinophyceae) isolates from Thailand based on morphology and phylogeny. *Phycol. Res.* **63**: 125–33.

- Ten-Hage, L., Turquet, J., Quod, J. P. and Couté, A. 2000. *Coolia areolata* sp. nov. (Dinophyceae), a new sand-dwelling dinoflagellate from the southwestern Indian Ocean. *Phycologia* **39**: 377–83.
- Wakeman, K. C., Yamaguchi, A., Roy, M. C. and Jenke-Kodama, H. 2015. Morphology, phylogeny and novel chemical compounds from *Coolia malayensis* (Dinophyceae) from Okinawa, Japan. *Harmful Algae* **44**: 8–19.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Detailed description of materials and methods.

Fig. S1. Micrographs of the Puerto Rican strain of *Coolia malayensis*.

Fig. S2. Confocal overlay image showing the thecal plates of the Brazilian strain of *C. malayensis* stained with Fluorescent Brightener 28.

Fig. S3. Maximum likelihood tree of *Coolia* spp. based on the ITS1-5.8S-ITS2.

Fig. S4. Line drawings of *C. monotis* and *C. malayensis*.

Fig. S5. Geographical distribution based on the gene sequence data of *C. monotis*, *C. malayensis*, *C. palmyrensis* and *C. santacroce*.