

## Article

# Morpho-Taxonomy and Molecular Characterization of *Coolia canariensis* S. Fraga and *Ostreopsis ovata* Fukuyo (Ostreopsidaceae, Dinophyceae) from Mauritius (Indian Ocean) Marine Coastal Waters

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**Abstract:** The marine dinoflagellate genera *Coolia* Meunier and *Ostreopsis* Schmidt have been reported in the Western Indian Ocean and include potentially harmful species. However, no comprehensive observations have been reported in Mauritian waters. The primary aim of this study was to isolate, identify and characterize potentially toxic epiphytic *Coolia* and *Ostreopsis* species from the coastal waters of Mauritius. Morphological characteristics were examined using light/fluorescence microscopy and scanning electron microscopy. The morphologies of the *Coolia* and *Ostreopsis* strains were similar to those of *Coolia canariensis* and *Ostreopsis ovata*. The phylogenetic analyses (large subunit ribosomal [LSU] rDNA D1/D2) revealed that the Mauritian strains of *Coolia canariensis* and *Ostreopsis ovata* clustered within the clades of these species complexes with other isolates from different areas. This represents the first record of *Coolia canariensis* in Mauritius, ascribed to *C. canariensis* phylogroups I and V, with the latter representing a new lineage of this species complex. The findings broaden the current body of knowledge of *Coolia canariensis* lineages, while the additional information of *Ostreopsis ovata* further supports the presence of an Indo-Pacific lineage.

**Keywords:** *Coolia canariensis* complex; epiphytic dinoflagellates; Mauritius; morphology; new records; *Ostreopsis ovata* complex; phylogeny



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## 1. Introduction

Benthic dinoflagellates thrive in shallow waters and have been observed as epiphytic, epibenthic and tychoplanktonic forms. Some benthic dinoflagellates are loosely attached to substrates and may become freely motile. Some species of *Gambierdiscus*, *Prorocentrum*, *Ostreopsis* and *Coolia* benthic dinoflagellates are known to produce different toxins, presenting public health and environmental risks [1]. Of these, *Coolia* was initially considered as a monotypic genus represented only by *Coolia monotis* Meunier (1919) and was described as a cosmopolitan species [2]. A second and third *Coolia* species were subsequently described, namely, *C. tropicalis* M.A. Faust [3] and *C. areolata* L. Ten-Hage, et al. [4], and both seem

to be restricted to tropical regions [5]. In the following years, a number of new taxa have been identified, and to date, five additional species of benthic *Coolia* have been described: *C. canariensis* S. Fraga [6], *C. malayensis* Leaw, et al. [7], *C. palmyrensis* Karafas, et al. [8], *C. santacroce* Karafas, et al. [8] and *C. guanchica* H. David, et al. [9]. Jeong, et al. [10] reported the presence of *C. canariensis* on Jeju Island in Korea; however, based on subsequent phylogenetic analysis, Momigliano, et al. [11] suggested the possible occurrence of a second cryptic species closer to *C. canariensis*. David, et al. [12] differentiated the clades as *C. canariensis* and *C. cf. canariensis*, which was followed by Karafas, et al. [8]. Recent studies recovered several phylogroups from the *C. canariensis* clade [13,14]. *Ostreopsis* was also considered as a monotypic genus represented only by *Ostreopsis siamensis* J. Schmidt, 1901. The genus *Ostreopsis* is under taxonomic revision, and presently, eleven species have been described: *Ostreopsis siamensis* J. Schmidt [15], *O. lenticularis* Y. Fukuyo [16], *O. ovata* Y. Fukuyo [16], *O. heptagona* D.R. Norris, et al. [17], *O. mascarenensis* Quod [18], *O. labens* M.A. Faust & S.L. Morton [19], *O. marina* M.A. Faust [20], *O. belizeana* M.A. Faust [20], *O. caribbeana* M.A. Faust [20] and, more recently, *O. fattorussoi* Accoroni, et al. [21] and *O. rhodesae* Verma, et al. [22].

Co-existing with other toxic dinoflagellate species from the genera *Prorocentrum* and *Gambierdiscus*, *Coolia* and *Ostreopsis* have been observed living in various benthic habitats such as macroalgae, dead corals and sediment [3,14,23–28]. A similar epiphytic habitat distribution has been observed in Mauritius [29–33]. These species are widely distributed in tropical, subtropical and temperate environments [34,35]. From the genus *Coolia*, *C. malayensis* presents a cosmopolitan distribution from tropical to temperate environments [28,36–38]. The type species *Coolia monotis* and *C. tropicalis* and *C. areolata* were originally described based only on their morphological features. *Coolia tropicalis* was re-described with genetic data provided by Mohammad-Noor, et al. [39]. The recently described species *C. canariensis*, *C. malayensis*, *C. palmyrensis*, *C. santacroce* and *C. guanchica* have been supported with phylogenetic analyses inferred from DNA sequence datasets and morphological data. *Ostreopsis* species present a widespread distribution in tropical and subtropical coastal marine habitats [40]. Of these, *Ostreopsis ovata* and *O. lenticularis* have been reported as the most widely distributed species [41]. *Ostreopsis* species have also been reported in temperate regions like the Northeast Atlantic coast, Mediterranean Basin, Southwest Pacific coast of New Zealand and Sea of Japan [42–49].

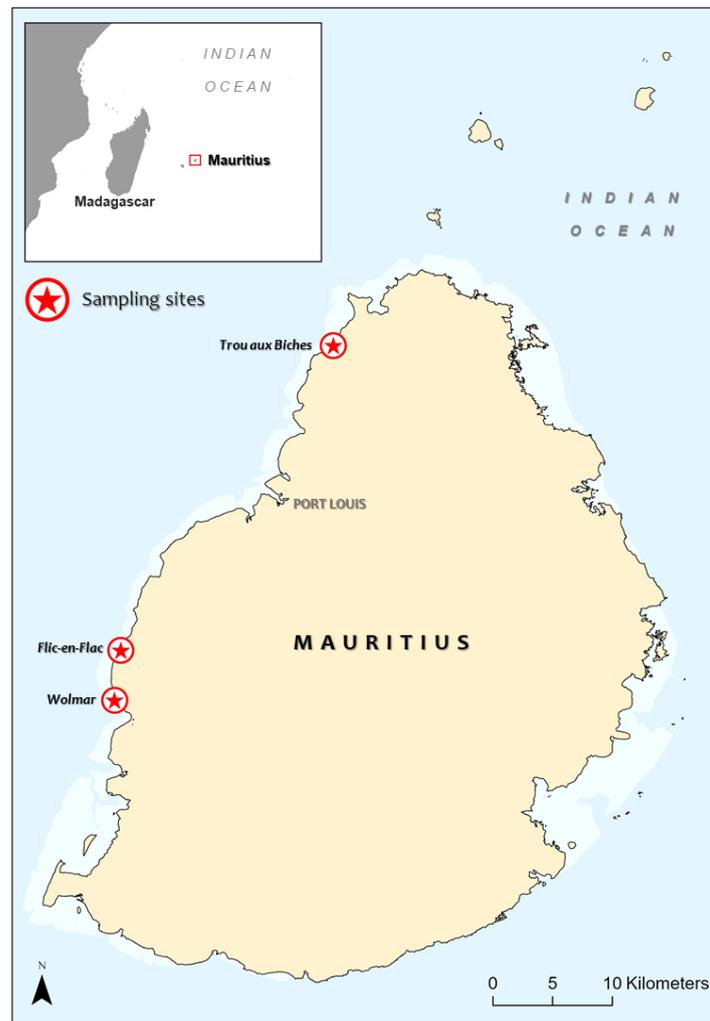
Previous studies of the Mascarene Islands indicated the presence of six species of *Ostreopsis* (*O. heptagona*, *O. labens*, *O. lenticularis*, *O. mascarenensis*, *O. ovata*, *O. siamensis*) and three species of *Coolia* (*Coolia monotis*, *C. tropicalis*, *C. areolata*) [4,18,29,50,51]. Of these, epiphytic Mauritian species of *Ostreopsis lenticularis*, *O. ovata*, *O. siamensis* and *Coolia monotis* together with an unidentified *Coolia* sp. have been reported [29,30]. Nevertheless, *Coolia monotis* appears to be restricted to the Northern Hemisphere [28]. Thus, previous records of *Coolia monotis* from Mauritius and the Western Indian Ocean [29] may likely represent other *Coolia* species. In this study, the taxonomic identity of potentially toxic epiphytic isolates from *Coolia* and *Ostreopsis* species were morphologically characterized using light/fluorescence microscopy and scanning electron microscopy, and phylogenetic relationships were inferred from the nuclear large subunit (LSU) rDNA D1/D2 region.

## 2. Materials and Methods

### 2.1. Study Area and Sample Collections

In this study, we conducted surveys during the summer period, and the sampling sites featured coarse to fine sand. At the time of sample collection, the sea surface temperature ranged between 26 °C and 33 °C, and salinity ranged from 38 to 40 ppt. Macrophyte samples, including macroalgae and seagrass, namely, *Turbinaria* sp., *Padina* sp., *Dictyopteris*

sp., *Syringodium* sp. and *Halodule* sp., not identified to species level were collected, between 0.5 and 1.0 m depth, from Wolmar (20°18'39" S, 57°21'56" E), Flic-en-Flac (20°16'22" S, 57°22'14" E) and Trou aux Biches (20°2'24" S, 57°32'25" E) in September 2015 (Figure 1).



**Figure 1.** Map showing the sampling sites in Mauritius.

Macrophyte samples were collected in 800 mL wide-mouthed plastic jars, filled with local seawater to approximately one-third and kept in the dark at an ambient temperature. In the laboratory, samples were vigorously shaken for approximately one minute and were sieved through 300, 125 and 20  $\mu\text{m}$  filters. Single cells of *Coolia* spp. and *Ostreopsis* spp. were isolated, from a mixed assemblage of dinoflagellates including *Prorocentrum* spp. and *Gambierdiscus* spp., using the micropipette technique [52] under a compound microscope. The *Coolia* and *Ostreopsis* cells were placed in individual wells of a 24-well plate filled with filter-sterilized f/2 medium [53] and incubated at 26 °C under an irradiance of 50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  on a 14 h/10 h light/dark cycle. Cell growth was monitored; when sufficient cell density was achieved after approximately 7 days, surviving isolates were transferred to 125 mL polycarbonate Erlenmeyer flasks containing 80–100 mL half-strength culture medium. Established cultures were sequentially transferred to full-strength medium and maintained at 26 °C, an irradiance of 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  in a 14 h/10 h light/dark cycle, without suspension.

Three *Coolia* strains (ISOC5, ISOC6 and ISOC40) and two *Ostreopsis* strains (ISO01 and ISO09) were successfully isolated and cultured. In this study, the modified Kofoidian

nomenclature system [54] as described by Besada, et al. [55] was used for naming the plates, thereby allowing comparisons with other genera of *Gonyaulacales* [56,57]. The tabulation applied here is apical pore complex (APC), 4', 6'', 6c, 6–7 s, 5''', 2'''''. The epitheca is composed of the APC, four apical plates (1'–4') and six precingular plates (1''–6''). The cingulum has six plates (c1–c6). The sulcus is made of six/seven sulcal plates. The hypotheca consists of five postcingular (1''''–5''') and two antapical (1''''–2''''') plates.

## 2.2. Microscopy

The cell morphology of the isolates was analyzed by light microscopy, epifluorescence microscopy and scanning electron microscopy following procedures similar to our earlier studies on the dinoflagellate species *Gambierdiscus caribaeus*, *Prorocentrum rathymum*, *P. fukuyoi* complex and *P. lima* complex [32,33]. Our findings are as detailed in Sections 3.1 and 3.2.

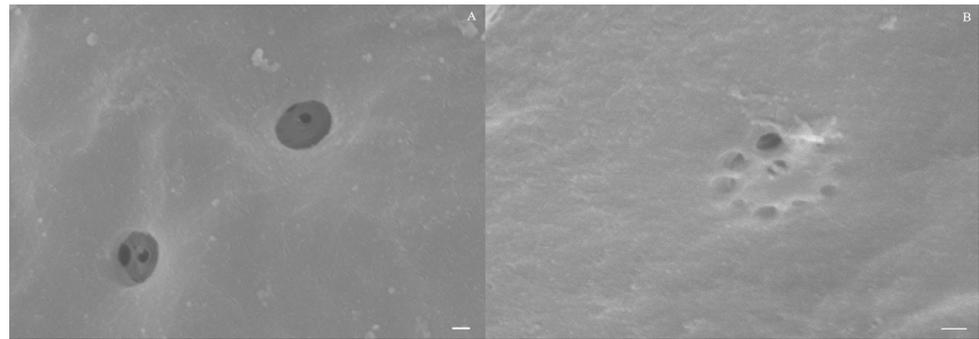
## 2.3. Nuclear DNA Extraction and PCR Amplification

Cells from approximately 5–10 mL of each clonal culture were harvested by centrifugation. Genomic DNA was extracted from the pellet using 10% Chelex<sup>®</sup> 100 [58]. Polymerase chain reaction (PCR) amplification for 25 µL reactions was performed using OneTaq<sup>®</sup> 2X Master Mix (New England Biolabs, Hitchin, Hertfordshire, UK), following the manufacturer's instructions with the primer pair D1R-F and D2C-R for the large subunit ribosomal (LSU) region [59]. Thermocycling conditions for *Coolia* were set at 95 °C for five minutes, followed by 35 cycles at 95 °C for one minute; annealing at 52 °C for 30 s; extension at 72 °C for two minutes, with a final extension at 72 °C for seven minutes. The thermocycling conditions of the *Ostreopsis* samples were set at 95 °C for five minutes, followed by 35 cycles at 95 °C for one minute; annealing at 52 °C for one minute; extension at 72 °C for two minutes, with a final extension at 72 °C for seven minutes. DNA sequencing was performed using the same primers as a commercial service (Inqaba, Pretoria, South Africa). The LSU sequences of *Coolia* and *Ostreopsis* strains were deposited in GenBank with the following accession numbers: ISOC5 (PP551266), ISOC6 (PP551267), ISOC40 (PP551268), ISO09 (PP551265) and ISO01 (PP551264).

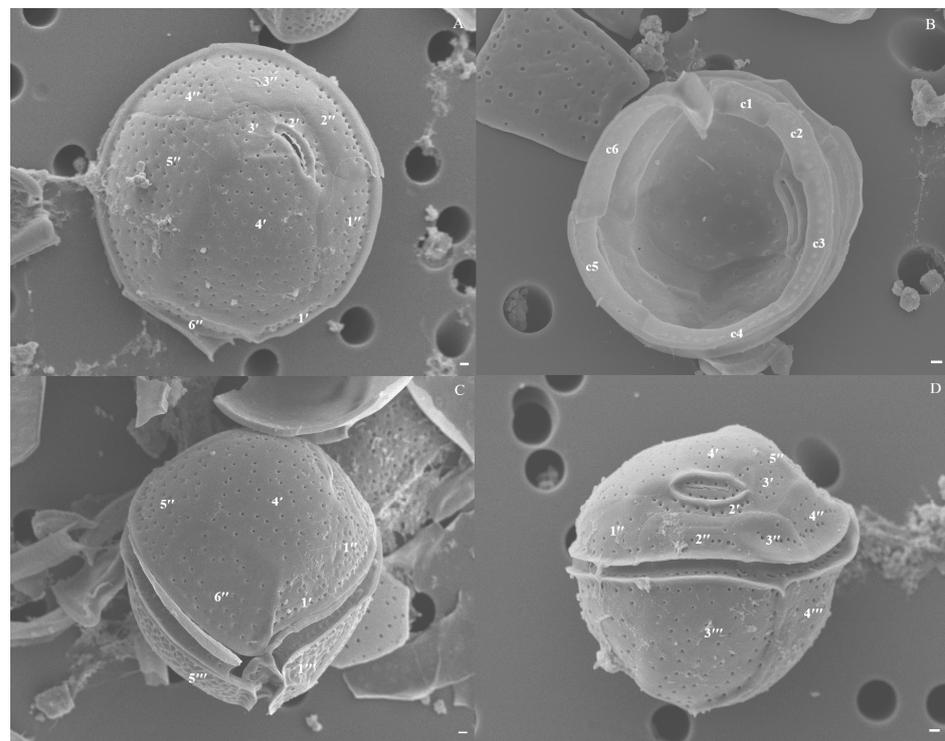
## 2.4. Phylogenetic Analyses

Nucleotide sequences of the D1/D2 LSU region obtained in this study were aligned using *Coolia* and *Ostreopsis* sequences downloaded from the GenBank database, respectively. Multiple sequence alignments were performed in Geneious Prime 2023.1.1 [60] using the Clustal Omega 1.2.2 plug-in. A model of evolution for the maximum likelihood (ML) method was performed in MEGA X version 10.1.7 [61]. In the LSU, a Tamura-Nei (TN93 + G) model for *Ostreopsis* and a Tamura 3-parameter (T92 + G + I) model for *Coolia* were selected. The optimal ML tree was subjected to 1000 bootstrap replicates. The phylogenetic relationships were also determined using the Bayesian inference (BI) method and, in this case, performed in the Geneious Prime platform via MrBayes 3.2.6. plug-in [62]. The best substitution models were obtained across the entire GTR model space. The Markov Chain Monte Carlo procedure was based on two independent trials with four chains each. Each chain was run for 2,000,000 generations, with sampling every 1000 generations. The first 10% of burn-in trees were discarded. Genetic distance (p-distance) was assessed using MEGA X. Our findings are as detailed in Sections 3.3 and 3.4.





**Figure 4.** Scanning electron micrographs of *Coolia canariensis* S. Fraga (ISOC5): (A) pore structure showing the internal sieve-like poroids; (B) inside view of thecal plate showing sieve-like poroids. Scale bars: (A,B) 100 nm.

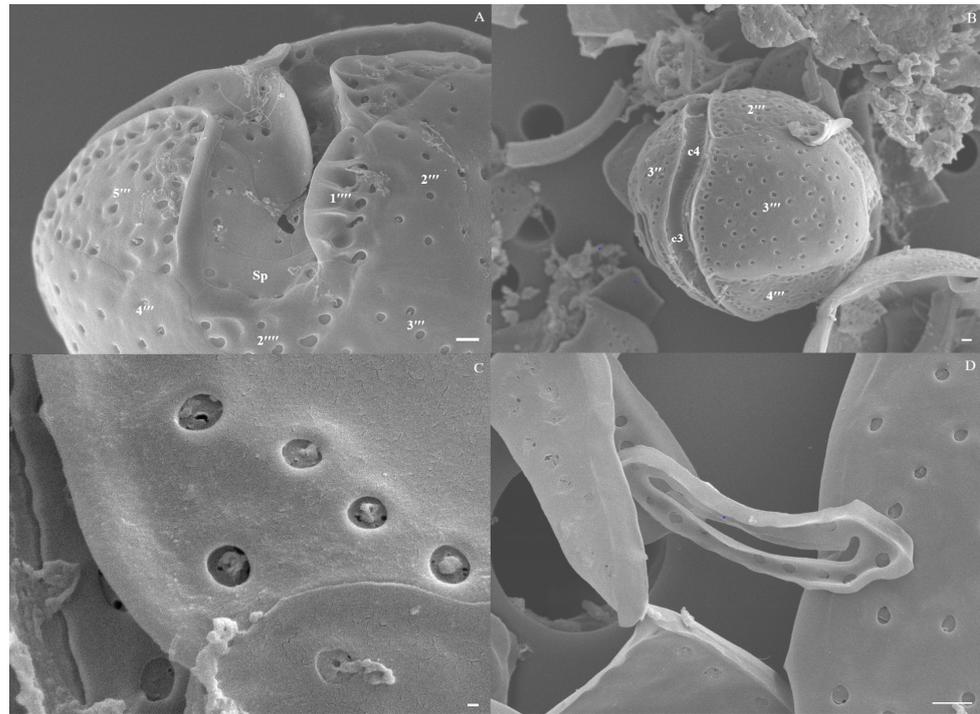


**Figure 5.** Scanning electron micrographs of *Coolia canariensis* S. Fraga (ISOC6): (A) apical view; (B) cingular plates; (C) oblique/ventral view; (D) dorsal view. Scale bars: (A–D) 1 µm.

The cell sizes of strain ISOC5 varied from 30 to 33 µm ( $33.2 \pm 2.7$  µm,  $n = 3$ ) in length, 25 to 33 µm ( $29.1 \pm 2.9$  µm,  $n = 3$ ) in width and from 29 to 34 µm ( $31.5 \pm 1.5$  µm,  $n = 5$ ) in depth. The cell sizes of strain ISOC6 varied from 24.8 to 32.5 µm ( $29.9 \pm 3.6$  µm,  $n = 3$ ) in length, 22.8 to 32.0 µm ( $28.8 \pm 4.2$  µm,  $n = 3$ ) in width and from 24 to 32 µm ( $27.5 \pm 3.4$  µm,  $n = 3$ ) in depth. It was not possible to observe all the sulcal plates (Figure 2C,D, Figures 3B and 6A).

The Po was elongated and varied in length from 7.5 to 12.5 µm ( $9.1 \pm 2.0$  µm,  $n = 4$ ) and from 6.5 to 7.2 µm ( $6.0 \pm 0.3$  µm,  $n = 3$ ) for the strains ISOC5 and ISOC6, respectively. The epitheca for both strains was slightly smaller than the hypotheca (Figures 2C,D and 5D). ISOC5 displayed a smooth epitheca with pores randomly distributed around the cell, except in plate 1'', where smooth to light ornamentation (small pits) (Figure 3A) and sieve-like poroids inside each pore (Figure 4A,B) can be observed. ISOC6 displayed a smooth epitheca with pores randomly distributed around the cell, except in plates 1' and 1'', where smooth to light ornamentation (Figure 6C,D) and poroids inside each pore

(Figure 6C) can be observed. Similarly, round and ovoid pores were observed in ISOC5 plate 4' (Figure 2A—ovoid pores, Figures 2C,D and 3A—round pores).



**Figure 6.** Scanning electron micrographs of *Coolia canariensis* S. Fraga (ISOC6): (A) oblique/ventral view; (B) antapical view; (C) detail of pores showing the internal poroids; (D) pore plate. Scale bars: (A,B,D) 1  $\mu$ m; (C) 100 nm.

The hypotheca for both strains displayed smooth to heavy ornamentation, except in plate 3''', where smooth to light ornamentation (small pits) can be observed for ISOC5 (Figure 3B,C), while for ISOC6, the plate was mostly smooth (Figures 5B and 6D). Light ornamentation (small pits) to heavy ornamentation (rugose) was observed in plates 1''' and 5''' (ISOC5: Figure 2C,D, ISOC6: Figures 5C and 6A). The cingulum was narrow (ISOC5 was  $2.0 \pm 0.6 \mu\text{m}$ ,  $n = 6$  and ISOC6 was  $1.8 \pm 0.5 \mu\text{m}$ ,  $n = 4$ ). The cingulum plates displayed both smooth to light ornamentation (ISOC5: Figures 2C,D and 3A,C and ISOC6: Figures 5C,D and 6B). Both strains displayed six cingular plates (ISOC5: Figure 3D and ISOC6: Figure 5B).

In both strains, the hexagonal plate 4' was the largest plate of the epitheca. Plate 4' was centrally positioned and surrounded by 1', 1'', 2', Po, 3', 5'' and 6'' (ISOC5: Figure 2C,D and ISOC6 Figure 5A,C). Similarly, in both strains, the first apical plate 1' was small and rectangular (ISOC5: Figures 2C,D and 3A and ISOC6: Figure 5A,C). In ISOC5, it was  $7.4 \pm 1.2 \mu\text{m}$  in length and  $4.2 \pm 0.7 \mu\text{m}$  in width, while in ISOC6, it was  $6.5 \mu\text{m}$  in length and  $4.2 \mu\text{m}$  in width. The apical plate 3' was small and pentagonal in shape. In ISOC5 and ISOC6, plate 3' connected to Po, 2', 4', 3'', 4'' and 5'' (ISOC5: Figure 2A and ISOC6: Figure 5A,D). The apical plate 2' was slightly curved and had an average length of  $13.1 \pm 1.4 \mu\text{m}$  (ISOC5) and  $9.6 \pm 1.1 \mu\text{m}$  (ISOC6).

The 6'' last precingular plate was nearly twice as wide and as long in ISOC5,  $12.7 \pm 1.2 \mu\text{m}$  in length and  $6.9 \pm 0.4 \mu\text{m}$  wide, while in ISOC6, it was  $13.6 \mu\text{m}$  in length and  $8.2 \mu\text{m}$  wide. The precingular plate 6'' of ISOC5 and ISOC6 had a W/L ratio of 1.8 and 1.6, respectively. The first precingular plate 1'' connected with plates 1', 2', 2'' and 4' (ISOC5: Figures 2C,D and 3A and ISOC6: Figure 5A,C). Plate 2'' was rectangular and connected with 2', 1'' and 3'' (ISOC5: Figures 2A and 3C and ISOC6: Figure 5A,D). Plate

3'' connected with 2'', 2', 3' and 4''. Plate 4'' connected with 3', 3'' and 5'', while plate 5'' connected with 3', 4', 4'' and 6'', and was the second largest plate in the epitheca (ISOC5: Figure 2A and ISOC6: Figure 5A,C).

All the hypothecal plates presented light to heavy ornamentation (ISOC5: Figures 2 and 3 and ISOC6: Figures 5 and 6). The first postcingular plate 1''' was triangular and connected with plates 2''' and 1'''''. It was the smallest among the postcingular plates and, together with the right edge of plate 5''', constituted the sulcal lists while defining the cingular displacement (ISOC5: Figures 2C,D and 3A and ISOC6: Figures 5C and 6A). The postcingular plates 2''', 3''', 4''', and 5''' were larger than plate 1'''. They displayed a radial distribution towards the antapex (ISOC5: Figure 3B,C and ISOC6: Figures 5D and 6B). Plate 1'''' was half oval-shaped and covered the anterior part of the sulcus (Figure 6A). Plate 2'''' was triangular (Figure 6A).

### 3.2. Morphology of Mauritian Strains of *Ostreopsis ovata*

The morphological features of wild live and fixed cells were examined from the sites of collection. The cells were broad ovoid, tear-shaped and ventrally tapering with the ventral area devoid of chloroplasts. In some cells, a tubular protrusion in the ventral area was observed (Figure 7A,C,E). The nucleus was located in the dorsal area and elongated chloroplasts were present. Flagella were not visible on live specimens. In the apical and antapical views, under different planes, scattered pores were observed on the thecal plates (Figures 7B,E and 8B,F). The dorsoventral (DV) length and width of the cells ( $n = 15$ ) were 58.52–69.21 ( $62.73 \pm 13.38$ )  $\mu\text{m}$  and 43.89–50.54 ( $47 \pm 2.48$ )  $\mu\text{m}$ , respectively. The DV length to width was 1.24–1.58 ( $1.32 \pm 0.12$ )  $\mu\text{m}$ .

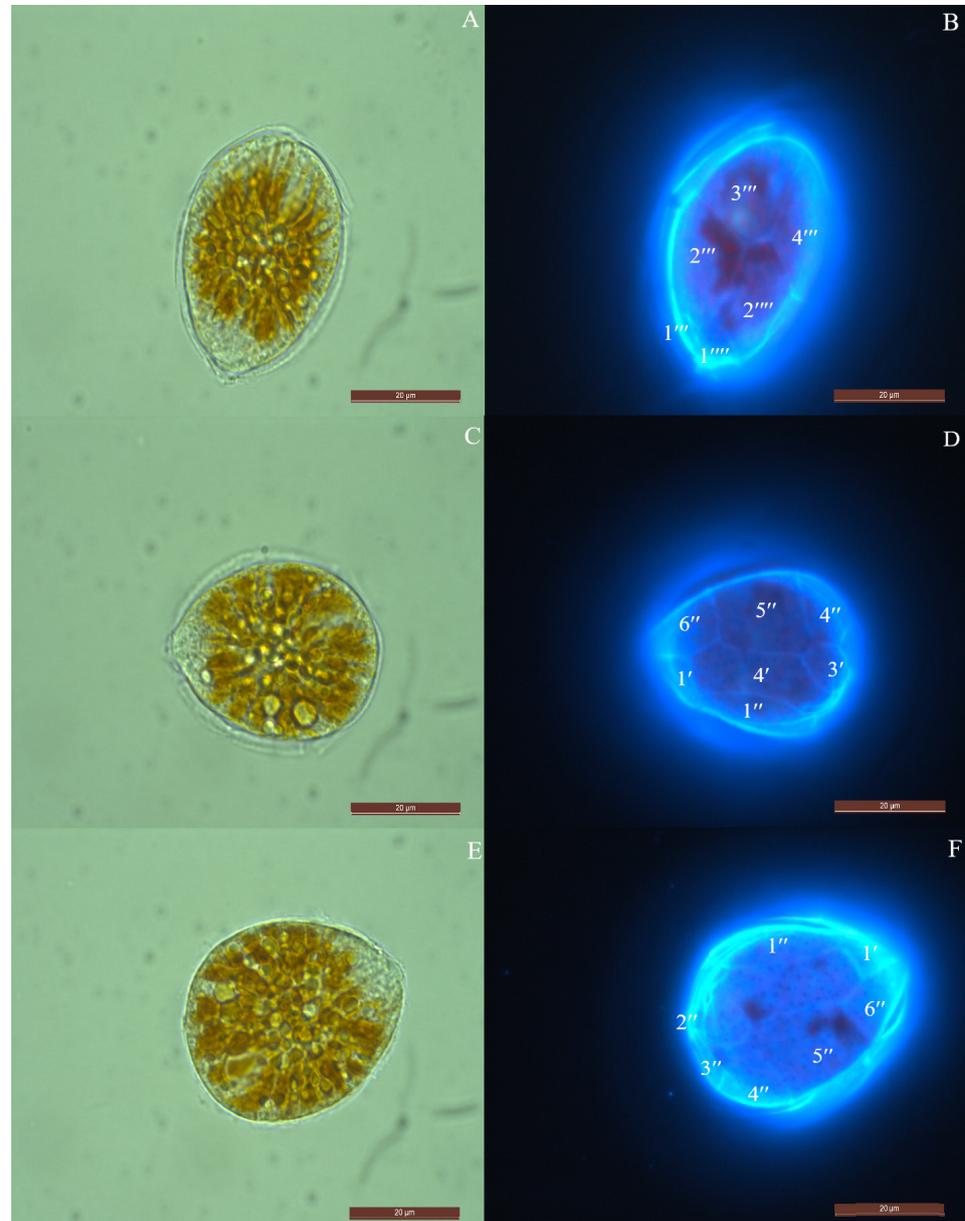
Under calcofluor staining, the thecal plates displayed the formula Po, 4', 6', 5''' and 2'''''. Plate 4' was large and hexagonal. It touched plates Po, 1', 2', 3', 2'', 5'' and 6''. The apical pore complex showed the same characteristic curvature of *Ostreopsis* species. Epithec plates are shown in Figures 7D,F and 8F. Plate 1' was quadrangular and connected with plates 4', 1'' and 6''. Plate 3' was pentagonal and touched plates 2', 4', 2'', 3'', 4'' and 5'' (Figures 7D,F and 8F). Plate 4' was large and hexagonal. It touched the plates Po, 1', 2', 3', 1'', 5'' and 6''. Plate 1'' was long and narrow. It is connected with plates 1', 2', 4' and 2''. Plate 2'' touched plates 2', 3', 1'' and 3''. Plates 3'' and 4'' were quadrangular, touched each other and were connected to plate 3'. Plate 4'' does not connect to plate 4'. Plate 5'' was wide and pentagonal, while plate 6'' was quadrangular. Regarding hypothecal plates (Figures 7B and 8B,E), the hypotheca had a central antapical plate 2'''''. It was connected with plates 1''', 2''', 3''', 4''', 5''' and 1'''''. Postcingular plates 3''' and 4''' were the largest, touching plates 2''' and 5''', respectively.

### 3.3. Phylogenetics of Mauritian Strains of *Coolia canariensis*

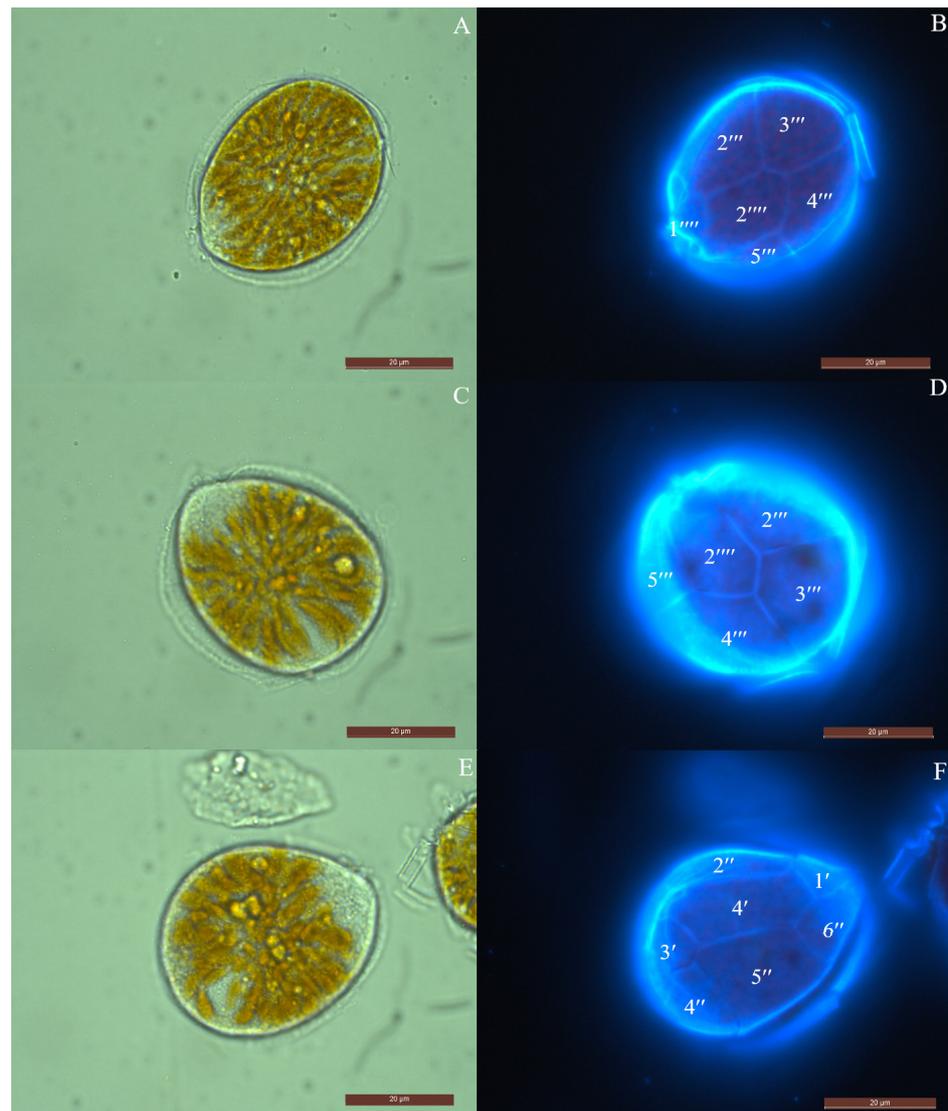
The final LSU data matrix [458 base pairs (bp) in length] included three *Coolia* strains isolated during this study, 55 *Coolia* strains and one sequence of *Ostreopsis ovata* as an outgroup. Bayesian inference (BI) and maximum likelihood (ML) based on LSU sequences generated similar trees. Seven distinct clades with high bootstrap and probability values were observed and represented currently recognized species.

DNA-based phylogeny depicts the Mauritian isolates in a clade containing *Coolia canariensis* strains (Figure 9). The *Coolia canariensis* clade constituted strongly supported values (98/1.0) and was closely related to the clade consisting of *C. guanchica* (strains KU514008 and KU514006). The *Coolia canariensis* clade contained four phylogroups. Strain ISOC5 showed support as a member of phylogroup I, consisting of six *C. canariensis* strains, including the holotype based on the strain VGO787 (GenBank [AM902738]) from Spain. *Coolia canariensis* phylogroup I (7 strains) differed by 7 bp (similarity 98.5%). Strains

ISOC6 and ISOC40 segregated as a separate phylogroup V with high bootstrap values and Bayesian posterior probability (91/0.97) and was sister to the large phylogroup III consisting of eighteen *C. canariensis* LSU identifiers, differing by 19–30 bp (similarities from 93.2 to 95.7%). Nevertheless, a wide nucleotide difference was observed between phylogroup II (ISOC6 and ISOC40) strains by 22 bp (similarity 95%).



**Figure 7.** Micrographs of wild *Ostreopsis* cf. *ovata* cells from Flic-en-Flac site. Light (A,C,E) and epi-fluorescent (B,D,F) microscopy micrographs. Apical and antapical views (A,C,E) showing light golden chloroplasts, elongated chloroplasts, nucleus visible as a gray body and ventral area devoid of chloroplasts. Apical and antapical views (B,D,F) showing the epithecal and hypothecal plates. Scale bars: (A–F) 20  $\mu$ m.



**Figure 8.** Micrographs of wild *Ostreopsis* cf. *ovata* cells from Trou aux Biches site. Light (A,C,E) and epi-fluorescent (B,D,F) microscopy micrographs. Apical and antapical views (A,C,E) showing light golden chloroplasts, elongated chloroplasts, nucleus visible as a gray body and ventral area devoid of chloroplasts. Apical and antapical views (B,D,F) showing the epithelial and hypothal plates. Scale bars: (A–F) 20 µm.

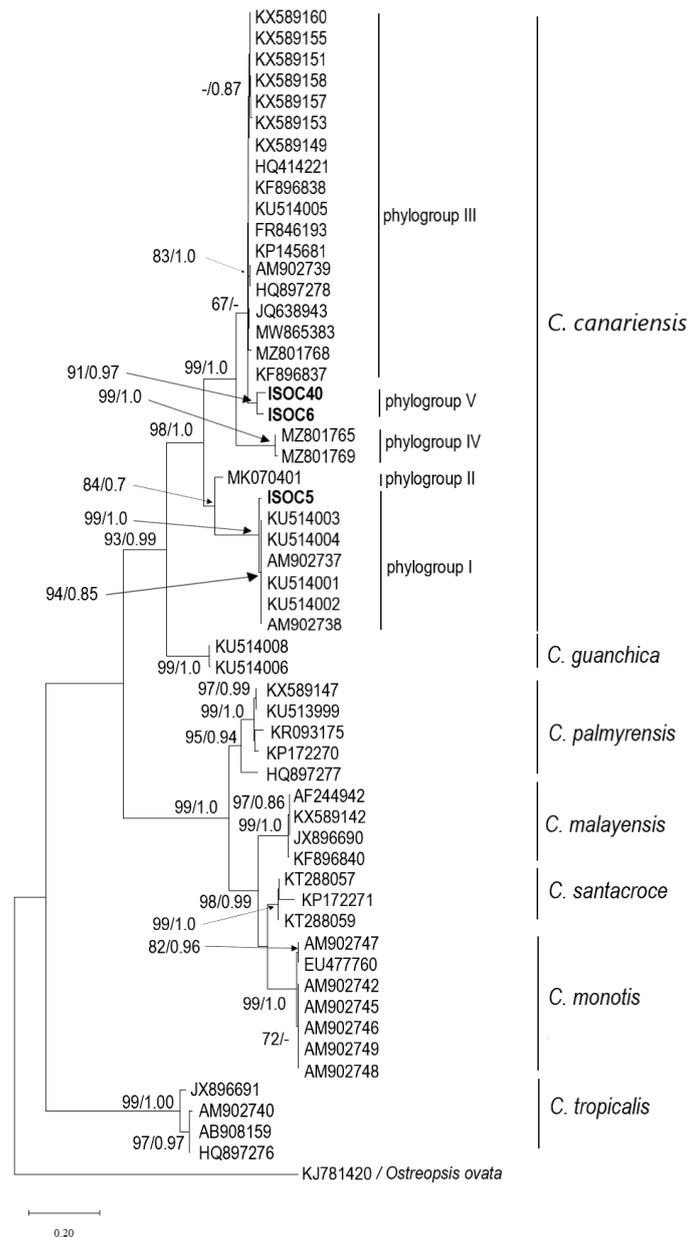
*Coolia canariensis* (30 strains) showed a rate intraspecific evolutionary divergence of 0.12, while for its closest species, *C. guanchica* (2 strains), the value was 0.03. The highest interspecific divergence was between *C. tropicalis* and *C. monotis* (0.71) and the lowest interspecific divergence was between *C. santacroce* and *C. malayensis* (0.13) (Table 1).

### 3.4. Phylogenetics of Mauritian Strains of *Ostreopsis*

LSU sequence data (D1/D2 region) of the Mauritian strains ISO01 and ISO09 were obtained. The final LSU data matrix together with other *Ostreopsis* species was 637 bp in length. The tree was rooted using a *Coolia monotis* strain.

When properly aligned, the DNA sequences of the Mauritian strains ISO01 and ISO09 were similar to *Ostreopsis ovata* LSU identifiers, AF244940 (Malaysia strain) and JX065571 (Vietnam strain), respectively, differing by 20 to 70 bp (similarities from 87.5 to 96.4%). They were also closely related to *O. ovata* LSU identifiers, namely, FM997921 and FM997924 (Indonesia strains), differing by 39 to 40 bp (similarities from 93.0 to 93.4%).

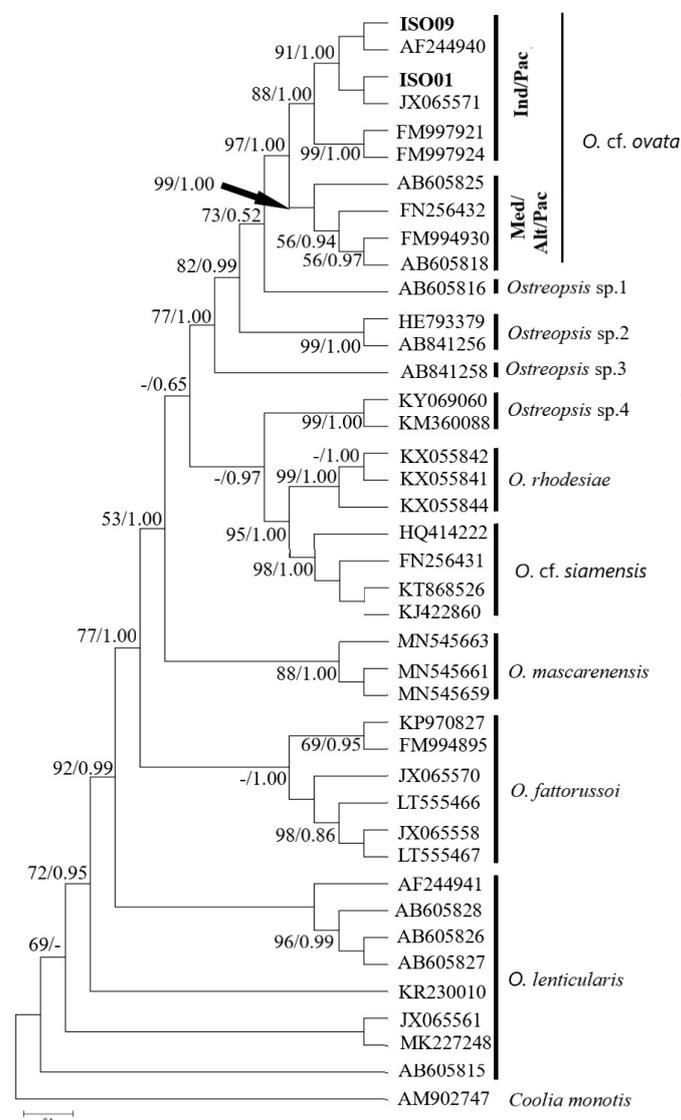
Bayesian inference (BI) and maximum likelihood (ML) based on LSU sequences generated similar trees. DNA-based phylogeny (Figure 10) depicts the Mauritian isolates in a sub-clade containing *O. ovata* strains closely related to those from Malaysia (AF244940), Vietnam (JX065571) and Indonesia (FM997921 and FM997924). This sub-clade was also closely related to another sub-clade containing *O. ovata* strains (AB605825—Japan strain, FN256432—Brazil strain, FM994930—Spain strain and AB605818—Japan strain). The *O. ovata* strains grouped together with maximum support (Bayesian posterior probabilities: 1.00; ML bootstrap support values: 97). This clade appeared as sister to *Ostreopsis* sp. 1 (1 strain), *Ostreopsis* sp. 2 (2 strains) and *Ostreopsis* sp. 3 (1 strain).



**Figure 9.** Molecular phylogenetic relationships of *Coolia* species inferred from partial large subunit (28S D1/D2 region) based on maximum likelihood and Bayesian inference analyses. Values at nodes represent ML bootstrap analyses and Bayesian posterior probability support, shown as ML/BI, respectively. ‘-’ represents unsupported value. Scale bar: number of nucleotide substitutions per site. Bold labels indicate sequences obtained in this study. Accession numbers: ISOC40 (PP551268); ISOC6 (PP551267); ISOC5 (PP551266).

**Table 1.** *Coolia* species: *C. canariensis* (1), *C. guanchica* (2), *C. palmyrensis* (3), *C. malayensis* (4), *C. santacroce* (5), *C. monotis* (6), *C. tropicalis* (7). Resulting net average distance shown as the number of base substitutions per site from averaging all sequence pairs between *Coolia canariensis* S. Fraga and its closest sister clades, are shown below the diagonal, and standard error estimate(s), are shown above the diagonal. In bold, the diagonal represents the within-the-clade divergences.

<i>Coolia</i> Clades	<i>C. canariensis</i>	<i>C. guanchica</i>	<i>C. palmyrensis</i>	<i>C. malayensis</i>	<i>C. santacroce</i>	<i>C. monotis</i>	<i>C. tropicalis</i>
<i>C. canariensis</i>	<b>0.12</b>	0.03	0.04	0.05	0.06	0.06	0.06
<i>C. guanchica</i>	0.24	<b>0.03</b>	0.04	0.05	0.05	0.05	0.06
<i>C. palmyrensis</i>	0.40	0.43	<b>0.01</b>	0.02	0.02	0.03	0.07
<i>C. malayensis</i>	0.50	0.50	0.19	<b>0.03</b>	0.02	0.02	0.07
<i>C. santacroce</i>	0.47	0.47	0.15	0.13	<b>0.00</b>	0.02	0.09
<i>C. monotis</i>	0.52	0.52	0.21	0.15	0.10	<b>0.00</b>	0.09
<i>C. tropicalis</i>	0.56	0.55	0.61	0.63	0.69	0.71	<b>0.05</b>



**Figure 10.** Phylogenetic analysis of the *Ostreopsis cf. ovata* (strains: ISO01 and ISO09) showing alignment of D1/D2 LSU rDNA sequences. Values at nodes represent ML bootstrap analyses and Bayesian posterior probability support, shown as ML/BI, respectively. ‘-’ represents unsupported value. Scale bar: substitutions per site. Bold labels indicate sequences obtained in this study. Accession numbers: ISO01 (PP551264); ISO09 (PP551265). Ind/Pac represents the Indo-Pacific. Med/Alt/Pac represent the Mediterranean Sea and the Atlantic and Pacific Oceans.

#### 4. Discussion

The Mauritian *Coolia* strains (ISOC5, ISOC6 and ISOC40) shared the general useful characteristics of the genus *Coolia* [3,6–9,12,14,37–39,63,64]. The morphological characteristics of *Coolia* species are antero-posteriorly compressed on an oblique apex/antapex axis relative to the cingulum plane.

In the original description of *Coolia canariensis* [6], cell length (27.2 to 38.4 µm) and width (25.6 to 40 µm) were distributed across the size variation of the Mauritian strains' length (24.8 to 32.5 µm) and width (22.8 to 32.0 µm). The size range of *Coolia canariensis* from previous records was 37 to 38.7 µm length and 28.4 to 43.6 µm width [65]; 24 to 35 µm length and 25 to 39 µm width [12]; 19.6 to 30.5 µm length and 23.8 to 29.3 µm width [13]; and 26 to 39 µm length and 28 to 39 µm width [38]. David, et al. [9] observed that the proportions of plate 6'' (7'' plate equivalent) (W/L ratio~1) of *Coolia guanchica* were relevant in delineating from *Coolia monotis* complex species including *C. monotis*, *C. malayensis*, *C. santacroce* and *C. palmyrensis*. The most apparent morphological difference between *Coolia monotis* and a closely related species is plate 5'' (6'' plate equivalent), which is the largest, and plate 4' (1' plate equivalent), which is to the left of the center, compared to *C. guanchica*, *C. canariensis*, *C. areolata* and *C. tropicalis*, where the epitheca 4'' (1' plate equivalent) is located centrally on the epicone [4,6,39]. David, et al. [9] further proposed thecal pores as an important diagnostic feature differentiating *C. guanchica* from all known species of the genus *Coolia*. They observed that the pores were larger in size and were present in higher numbers in plates 4' (1' plate equivalent), 6'' (7'' plate equivalent) and 3'''. They further observed that the lighter to void ornamentation of the hypotheca differentiated *C. guanchica* from *C. canariensis* and *C. areolata*.

Based on the phylogenetic analyses using the LSU (D1/D2), the overall relationships of *Coolia* species were congruent with findings from previous studies [6–8,11,12,14,28,36–39]. The genetic data presented here for Mauritius strains of *Coolia* indicate a close relationship to *C. canariensis*. Currently, from *C. canariensis* species complex, phylogroup IV has been reported in the Pacific Ocean, phylogroup III has been reported in the Atlantic and Pacific Oceans, while phylogroups I and II have been reported in the Atlantic Ocean [14]. Thus, ISOC6 and ISOC40 represent the first phylogroup V in the Western Indian Ocean (Figure 9). Strain ISOC5 presented a broader distribution of phylogroup I (Figure 9). It is worth noting that a clade which is morphologically close to *Coolia canariensis*, *C. areolata*, was described on Réunion Island, approximately 230 km southwest of Mauritius. However, no DNA sequences are currently available for *C. areolata*. Our phylogenetic and genetic divergence analyses support the cryptic nature of *Coolia canariensis*.

*Ostreopsis* species have been mainly described in terms of their cell morphology and thecal plate pattern. However, most of the *Ostreopsis* species have been reported with a similar thecal plate pattern and bear morphological plasticity. Thus, species delineation for this genus remains unclear. Some of the current studies have re-investigated previously described *Ostreopsis* species from or near the type localities, with emphasis on both morpho-taxonomy and molecular characterization. *Ostreopsis lenticularis* and *O. siamensis* have recently been re-described by Chomérat, et al. [66] and Nguyen-Ngoc, et al. [67]. Similarly, *Ostreopsis mascarenensis* with type localities of the Mascarene Islands (including Rodrigues, Mauritius and Réunion islands) has been re-described for Réunion Island Chomérat, et al. [68].

In this study, the morphology was described for *Ostreopsis* cells obtained from field samples. The development of aberrant morphologies in cultures has been recorded by other researchers and likewise observed in this study. In this study, some of the cells grown in culture had the characteristic form of *Ostreopsis*. Nevertheless, they were of limited use for morphological identification. The phenotypic traits of the field specimens from

Mauritius indicated that they belonged to the genus *Ostreopsis* (e.g., shape, size and thecal plate pattern). Presumably, the field samples belong to the same species, bearing in mind that in the field, different species might co-exist. The DNA-based phylogeny depicts the Mauritian isolates in a clade containing *Ostreopsis* cf. *ovata* strains. Past studies indicated the cell shape of *Ostreopsis* cf. *ovata* species as variable with ovate, oblong, narrow-ovoid, tear-shaped, oval-tear-shaped, ovoid-oblong and drop-shaped aspects ([43] and references therein). In the original description of *Ostreopsis* cf. *ovata* [16], the length (50 to 56  $\mu\text{m}$ ) and width (25 to 35  $\mu\text{m}$ ) were larger than the size variation of the Mauritian strains' length (24.8 to 32.5  $\mu\text{m}$ ) and width (22.8 to 32.0  $\mu\text{m}$ ). However, in comparison, the size range [dorsoventral length (DV): width (W)] of the Mauritian field samples was larger than previous records for the region and those for distant geographical localities—Mascarene Islands (DV: 47–55  $\mu\text{m}$ ; W: 27–35  $\mu\text{m}$ ) [29], Réunion Island (DV:  $53 \pm 6.9$   $\mu\text{m}$ ; W:  $37.7 \pm 5.6$ ) [51], South China Sea (DV:  $47.5 \pm 3.1$   $\mu\text{m}$ ; W:  $37.1 \pm 3.3$ ) [69], Korea (DV: 35.9  $\mu\text{m}$ ; W: 26.4) [44], Thailand (DV:  $35.1 \pm 3.2$   $\mu\text{m}$ ; W:  $24.5 \pm 2.0$   $\mu\text{m}$ ) [70], Japan (DV: 28.1  $\mu\text{m}$ ; W: 21.2  $\mu\text{m}$ ) [71], Caribbean Sea (DV: 41.3  $\mu\text{m}$ ; W: 35.2  $\mu\text{m}$ ) [55], Egypt (DV: 29.5  $\mu\text{m}$ ; W: 27.2  $\mu\text{m}$ ) [72], Brazil (South Atlantic Ocean) (DV: 35–65  $\mu\text{m}$ ; W: 20–40  $\mu\text{m}$ ) [73] and Gulf of Gabès (southeastern Mediterranean Sea) (DV: 27–65  $\mu\text{m}$ ; W: 19–57  $\mu\text{m}$ ) [74]. The Mauritian field samples were within the size range of isolates from the Atlantic Iberian Peninsula (DV:  $69.6 \pm 7$   $\mu\text{m}$ ; W:  $44.72 \pm 6.3$   $\mu\text{m}$ ) [43]. This is in agreement with Hua, et al. [69], as cell size might not be reliable for species delineation due to its plasticity. In addition, in the present study, the Mauritian field samples exhibited the thecal pattern attributed to the genus *Ostreopsis*.

The phylogeny analysis based on the LSU sequence obtained from 40 isolates of *Ostreopsis* spp. confirms that the strains isolated during this study were *O. cf. ovata*. Maximum likelihood analysis and Bayesian inference based on the LSU (D1/D2) region showed that the *O. cf. ovata* sub-clade was clearly separated, with high ML and posterior probability (BI) values, consistent with recent studies [68,69,75]. In this study, the *O. cf. ovata* tropical sub-clade consisted of the Mauritian, Malaysian, Vietnamese and Indonesian strains, and represented the Indo-Pacific strains. Similarly, the second well-supported *O. cf. ovata* temperate/subtropical sub-clade consisted of strains derived from Mediterranean, Pacific and Atlantic localities. This is in agreement with several past studies [5,51,69–71,76,77].

## 5. Conclusions

In this study, morphological and phylogenetic data concerning potentially toxic epiphytic *Coolia canariensis* and *Ostreopsis ovata* species are presented. In summary, based on the morphological and molecular data, two lineages of *Coolia canariensis* species complex are documented from the coastal waters of Mauritius. Similarly, the *Ostreopsis* isolate represented the first *O. ovata* species characterized based on morphological and molecular data from Mauritius and supported the presence of an Indo-Pacific lineage.

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