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Chloroplasts morphology investigation with diverse microscopy approaches and inter-specific variation in *Laurencia* species (Rhodophyta)

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The present study described with different microscopy approaches chloroplasts lobes in *Laurencia* sensu latu (Rhodophyta) species and found inter-specific differences among them. Chloroplasts were investigated using confocal laser scanning microscopy (LSM), transmission electron microscopy (TEM) and high resolution scanning electron microscopy (HRSEM). Using and TEM and HRSEM images we distinguished chloroplasts with lobes than chloroplasts without lobes in *Yuzurua poiteaui* var. *gemmifera* (Harvey) M. J. Wynne and *Laurencia dendroidea* J. Agardh cortical cells. The LSM images showed chloroplasts lobes (CLs) with different morphologies, varying from thicker and longer undulated projections in *Y. poiteaui* var. and *L. dendroidea* to very small and thin tubules as in *Laurencia translucida* Fujii & Cordeiro-Marino. The diameter and length of CLs from *Y. poiteaui* var. and *L. dendroidea* were significantly higher than *L. translucida* CLs ($p < 0.01$). Based on LSM observations, we suggest that lobes morphology has a taxonomic validity only to characterize *L. translucida* species.

Key Words: chloroplasts lobes; confocal laser scanning microscopy; *Laurencia dendroidea*; *Laurencia translucida*; morphometry; transmission electron microscopy; *Yuzurua poiteaui* var. *gemmifera*

INTRODUCTION

Chloroplast lobes (CLs) are chloroplasts extensions filled with thylakoid membranes, found in polymorphic and dividing chloroplasts of plant and algae (Barnabas 1982, Sarafis 1998, Wilson et al. 2002, Škaloud et al. 2005, Proctor et al. 2007, Tischendorf et al. 2007, Schottkowski et al. 2012). Although CLs were already described in different red algae classes such as Cyanidiophyceae (Tischendorf et al. 2007), Bangiophyceae (Bisalputra and Bailey 1973, Cole and Sheath 1980) and Florideophyceae (Young 1978), their ultrastructure remains poorly know in red algae.

Recent morphology studies in red algae addressed the

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cellular responses of chloroplasts (e.g., CLs) exposed to different environmental conditions (Bouzon et al. 2012, Schmidt et al. 2012, Gouveia et al. 2013, Dos Santos et al. 2014). On other hand, ultrastructure of CLs have been recognized as an important tool in red algae taxonomy and phylogeny (Kikuchi and Shin 2005, Müller et al. 2010, Kushibiki et al. 2012), even this characteristic being quite variable (Cole and Sheath 1980, Wilson et al. 2002, Gillard et al. 2008). Thus, the study of CLs by using different microscopy approaches should improve the basic knowledge about this organelle in different red macroalgae species.

The *Laurencia* complex (Florideophyceae) contains 208 species accepted taxonomically being separated into seven genera: *Laurencia*, *Osmundea*, *Chondrophycus*, *Palisada*, *Yuzurua*, *Laurenciella*, and *Coronaphycus* (Metti et al. 2015). *Laurencia* species have been reported worldwide, occurring from intertidal to subtidal zones up to 65 m in depth and from temperate to tropical shores (Fujii et al. 2011).

Many species from *Laurencia* complex have no defined taxonomic boundaries and present extensive morphological plasticity, making their taxonomic delimitation difficult (Fujii et al. 2011). Morphological characters (Saito 1967, Nam et al. 1994, Garbary and Harper 1998, Nam 1999, 2006) and molecular markers (Nam et al. 2000, Abe et al. 2006, Fujii et al. 2006, Díaz-Larrea et al. 2007, Gil-Rodríguez et al. 2009, Martin-Lescanne et al. 2010) have been used for delimiting taxa, but until know no study performed the cellular characterization of CLs in *Laurencia* species.

Laurencia species are prolific synthesizers of halogenated terpenes and acetogenins (Fujii et al. 2011). These metabolites play important ecological roles, but also many biotechnological applications were described for them, e.g., antifouling paint (Da Gama et al. 2003). In addition, the genomic information of *L. dendroidea* species had advanced in consequence of a broad transcriptomic analysis, which unveiled many genes responsible for the synthesis of terpenoid compounds (De Oliveira et al. 2012, 2015).

It is well documented that *Laurencia* species, which stores halogenated metabolites inside storage structures as *corps en cerise* (CC) (Feldmann and Feldmann 1950), produces a higher quantity of halogenated metabolites than species without CC (Suzuki et al. 1987). However, studies with a cellular approach are scarce. Until now, among all species from *Laurencia* complex, only two have been extensively investigated in cellular, chemical and ecological levels, *Laurencia dendroidea* J. Agardh (Sudatti et al. 2006, Salgado et al. 2008, Paradas et al. 2010) and *Laurencia translucida* Fujii & Cordeiro-Marino (Paradas 2013).

Concerning major advances in cell biology, it was shown that *L. dendroidea* transports halogenated metabolites from CC to thallus surface in response to epiphytic bacteria through vesicle traffic (Paradas et al. 2010). In addition, Reis et al. (2013) showed that cytoskeleton elements have a central role in this *L. dendroidea* defensive system. In this case, it was shown that microfilaments transport the halogenated metabolites within vesicles by membranous tubular connections from CC to the cell periphery, while microtubules are involved in the vesicle positioning along cell periphery (Reis et al. 2013).

In this way, the aim of this work was to perform a fine characterization of the CLs in three species of *Laurencia* sensu latu (*Yuzurua poiteaui* var. Harvey M. J. Wynne, *L. dendroidea*, and *L. translucida*) using confocal laser scanning microscopy (LSM), conventional transmission electron microscopy (TEM), and high resolution scanning electron microscopy (HRSEM).

MATERIALS AND METHODS

Algal sampling

Y. poiteaui var. gametophytes were collected by self-contained underwater breathing apparatus (SCUBA) in the subtidal (December 2011) of Sprigger Bank, Florida Keys (Florida State, USA; 22°45′42.17″ N, 41°52′29.25″ W, 2 m depth, WC Paradas, *Y. poiteaui* var., RB 630073), while gametophytes of *L. translucida* and *L. dendroidea* were harvested from the lower intertidal zone at Rasa Beach (Armação dos Búzios, Rio de Janeiro State, Brazil; 22°43′58″ S, 41°57′25″ W, rocky shore, WC Paradas, *Laurencia translucida* RB 629414 and *L. dendroidea* RB 629412) in January of 2012. No specific permit was required for collection of *Y. poiteaui* var., *L. dendroidea*, and *L. translucida* samples in the studied areas. The harvesting areas are public and the algae are not endangered or protected species. These samples were transported alive to the laboratory in local seawater inside thermal containers.

LSM

Live algae (*Y. poiteaui* var., *L. dendroidea*, and *L. translucida*) were sectioned longitudinally with a razor blade, placed on glass cell chambers covered with coverslips (Corning Inc., Corning, NY, USA), and then immediately

observed under a Leica TCS SPE AOBS (Leica Microsystems Company, Wetzlar, Hesse, Germany) or a Zeiss 710 LSM (Zeiss Company, Oberkochen, Baden-Württemberg, Germany). We took advantage of the natural property of light absorption and the auto-fluorescence of chlorophyll *a* to obtain images of chloroplasts and their lobes. Imaging was performed using the violet laser (at 405 nm) as the excitation wavelength and the red light (650-750 nm) as emission. Images were collected with 2.048 × 2.048 pixels of resolution using LASER at low potency and faster scanning speed to avoid pigment bleach during the acquisition.

Morphometry of CLs

The diameter and length of CLs from *Y. poiteaui* var. *L. dendroidea*, and *L. translucida* vegetative cortical cells were measured using LSM images and ImageJ software (Abramoff et al. 2004). The data were evaluated for similarity of variance using Levene's test (ANOVA). One-factor analyses of variance (ANOVA) were performed to compare the mean values of CLs diameter and length randomly from 6 cells from different gametophytes (n = 10 per cell, total n = 60) by using STATISCA ver. 8.0 software (Statsoft Inc., Tulsa, OK, USA). A Tukey *post-hoc* test was employed to compare the diameter and length of *Laurencia* species CLs after a significant ANOVA test. Differences were considered significant when $p < 0.05$.

Sample preparation for TEM

For ultrastructural characterization of CLs, *Y. poiteaui* var. individuals were fixed with 4% formaldehyde and 2.5% glutaraldehyde in a seawater containing 25 mM piperazine-N,N´-bis 2-ethanesulfonic acid (PIPES) and 25 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) for 2 h at room temperature (all reagents were obtained from Sigma-Aldrich, St. Louis, MO, USA) and washed in the same buffer (3 × 10 min). Afterwards, the samples were submitted to increasing series of glycerol (10, 20, and 30%) in buffered seawater for 12 h each concentration. Thereafter, the samples were transferred to flat aluminum supports and fast-plunged into liquid freon 22 cooled with liquid N_2. Frozen fragments of *Y. poiteaui* var. were freeze-substituted with 1.5% uranyl acetate in methanol at -90°C for 24 h using a freeze-substitution machine (Leica Microsystems Company) (Kachar and Reese 1988). Samples were embedded in acrylic resin (Lowicryl) and polymerized under UV light for 2 days at -45°C.

Ultrathin sections (50 nm) were obtained in a Reichert-Jung ultramicrotome (Leica Microsystems Company), collected on copper grids (300 mesh) (Electron Microscopy Sciences, Hatfield, PA, USA) and observed in a JEOL 1010 EX TEM microscope operated at 80 kV (Jeol Company, Tokyo, Japan).

Sample preparation for HRSEM

Fragments of thalli from *L. dendroidea* and *Y. poiteaui* var. were cut with a razor blade in random directions to expose the cytoplasm and immediately fixed with 4% formaldehyde, 2.5% glutaraldehyde, 25 mM PIPES, 25 mM HEPES, 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 µM taxol, and 2 µM phalloidin in distilled water (pH 7.4) for 30 min at room temperature (all reagents were obtained from Sigma-Aldrich). The post-fixation step was performed following the osmium tetroxide-thiocarbohydrazide-osmium tetroxide (OTOTO) method, as follows: alternated bathes of 1% osmium tetroxide and 1% tannic acid aqueous solutions for 1 h each with washes in between (Kachar et al. 2000). Then, the samples were dehydrated with an ethanol series (30, 50, 70, and 100%), critical-point dried and mounted on aluminum holders coated with carbon conductive tape. The thalli of *L. dendroidea* and *Y. poiteaui* var. was physically fractured accordingly previous works (Paradas et al. 2010, 2015). Then, the samples were coated with a thin platinum layer (6 nm) using a Balzers BAF 300 freeze-fracture machine (Oerlikon, Balzers, Liechtenstein). Images were captured in a field emission gun SEM Hitachi 4800 (Hitachi Ltd., Tokyo, Japan; at 5 kV, spot-size = 2 nm, and working distance = 2 mm).

RESULTS

LSM images of *Y. poiteaui* var. cortical cells revealed an auto-fluorescence pattern (~700 nm) related to chlorophyll *a* signal in chloroplasts adjacent to the plasma membrane (Fig. 1A). No other organelle presented red auto-fluorescence. The chloroplasts observed exhibited CLs as tubular projections that connected series of chloroplasts (Fig. 1A, inset). The average diameter and length of the tubules were 0.47 ± 0.35 µm and 4.52 ± 4.30 µm, respectively (Table 1).

Laurencia dendroidea HRSEM images of cortical cells showed numerous chloroplasts and smaller spherical mitochondria adjacent to the plasma membrane (Fig. 1B). The chloroplasts exhibited elongated shapes with lobes

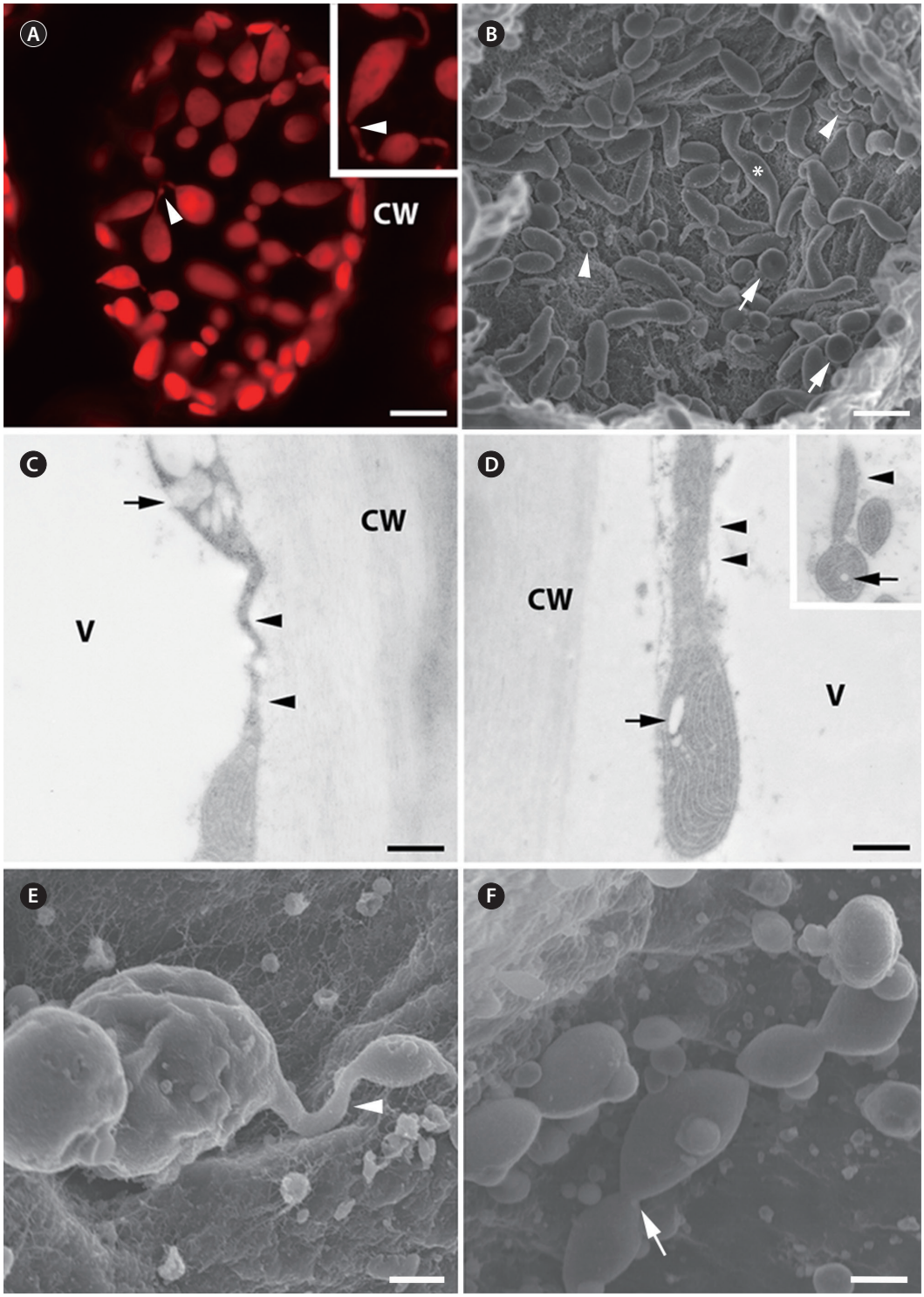

Fig. 1. Choroplast lobes (CLs) in *Laurencia* cortical cells. (A) A confocal laser scanning microscopy mage of *Yuzurua poiteaui* var. showing the auto-fluorescence of chloroplasts. The insert image shows the detail of chloroplasts connected by tubular projections (arrowheads). CW, cell wall. (B) A high resolution scanning electron microscopy (HRSEM) image of *L. dendroidea* elongated lobed (asterisk) and discoid chloroplasts (arrows). Arrowheads are ponting to mitochondria. (C) A transmission electron microscopy (TEM) image of *Y. poiteaui* var. showing a chloroplast without lobes and thin cytoplasm projection (arrowheads). Arrow indicates starch grains. V, vacuole. (D) A TEM image of *Y. poiteaui* var. showing the CLs with thylakoids membranes (arrowheads) growing from small plastids. Arrows are pointing to chloroplasts inclusions between thylakoids membranes. (E) A HRSEM image of *Y. poiteaui* var. showing the CL (arrowhead). (F) Image of the *Y. poiteaui* var. cytoplasm with attached chloroplasts (arrow). Scale bars represent: A, 4 μm; B, 5 μm; C, 2 μm; D, 1.5 μm; E, 750 nm; F, 850 nm.

or discoid shapes without lobes (Fig. 1B). In the same way, TEM images of *Y. poiteaui* var. cortical cells were capable to differentiate chloroplast without lobes (Fig. 1C) from chloroplasts with lobes (Fig. 1D, inset). HRSEM of *Y. poiteaui* var. cortical cells showed a different perspective of the chloroplast tubular lobes as described early by LSM and TEM (Fig. 1E). It is clear that the projections are part of the chloroplast *per se*, and not part of the cytoplasm or other organelle. Often, we observed chloroplasts fused laterally as shown in Fig. 1F.

Three-dimensional reconstructions of *Y. poiteaui* var. whole cells performed by LSM revealed that, in many instances, chloroplasts were fused laterally and formed a row of several chloroplasts giving a unity aspect (Fig. 2A). Closer look at the *L. dendroidea* chloroplast by LSM showed the details of CLs with long projections connecting the plastids (Fig. 2B). The CLs exhibited different morphologies, varying from thicker and longer undulated projections as in *Y. poiteaui* var. (Fig. 1A, inset) and *L. dendroidea* (Fig. 2B-D) to very small and thin tubules as in *L. translucida* (Fig. 3A-D). The diameter and length of CLs from *Y. poiteaui* var. and *L. dendroidea* were significantly higher than *L. translucida* CLs ($p < 0.01$). Since chlorophyll is specifically found into the plastids, all red signals from LSM are assumed to come from chloroplasts.

DISCUSSION

The goal of the present study was describe CLs with different microscopy approaches in *Laurencia* sensu latu (Rhodophyta) species and find inter-specific differences among them. CLs were observed by HRSEM, specifically in *Y. poiteaui* var. and *L. dendroidea* cortical cells. The HRSEM images of both algae cortical cells differentiated CLs profile from only associated chloroplasts. The CLs were also visualized in the *Y. poiteaui* var. cortical cells by LSM and TEM, while in *L. dendroidea* and *L. translucida* cortical cells by LSM.

Recent studies showed the importance of red algae CLs for species delimitation (Müller et al. 2010, Kushibiki et al. 2012). For example, Kushibiki et al. (2012), based on TEM and LSM images showed that *Bulboplastis apyrenoidosa* (Rhodellophyceae) could be distinguished from *Rhodospora sordida* (Rhodellophyceae) by the shape of its chloroplasts, while *Bulboplastis* has a single lobed chloroplast, *Rhodospora sordida* has multiple discoid chloroplasts (Geitler 1927, Johansen et al. 2005). Ultrastructural of CLs were also used by Yoon et al. (2006) to proposed three monophyletic classes: Rhodellophyceae, Porphyridiophyceae, and Stylonematophyceae.

Škaloud et al. (2005) based on LSM images found differences among CLs morphology in different ontogeny phases from *Dictyochloropsis* species (*D. splendida*, *D. reticulate*, and *D. symbiontica*) (Trebouxiophyceae). Laser scanning confocal microscopy has also been repeatedly applied for the investigation of chloroplast morphology and structural dynamics in higher plants (Pyke and Page 1998, Sarafis 1998, Zheng et al. 2002). Sarafis (1998) observed CLs, by LSM images, in the Angiosperm *Ophiopogon nigrescens* (Asparagaceae) and in the Anthocerophyta *Notothylas* sp. (Notothyladaceae), and established a close morphological relation between CLs and stromules. Stromules or stroma-filled tubules are structures constituted of stroma found in green algae and plants, which are involved in molecular transport between chloroplasts and other organelles (Menzel 1994, Hanson and Sattarzadeh 2011).

At the present work, the diameter and length of CLs found in *Y. poiteaui* var. (0.47 ± 0.10 µm / 4.52 ± 1.0 µm) and *L. dendroidea* (0.47 ± 0.05 µm / 6.6 ± 2.0 µm) presented similar measurements from that determined in plant stromules (diameter 0.35-0.85 µm / length up to 220 µm) (Natesan et al. 2005). But, stromules are free of thylakoids (Hanson and Sattarzadeh 2011), while *Laurencia* CLs have chlorophyll *a* and perform photosynthesis. Further studies using specific stroma dyes are needed to verify the presence of stromules in *Laurencia* species.

The LSM images of live cortical cells from *Laurencia* sensu latu species showed that CLs of each species has its own characteristics. Generally, all of them are lobed, but with a certain kind of idiosyncrasy. For example, *L. dendroidea* and *Y. poiteaui* var. CLs are in general undulated varying from thicker / longer to small / thin tubules. Otherwise, the *L. translucida* CLs are always thin and short like inconspicuous projections. The *L. dendroidea* and *Y.*

Table 1. Morphometric analyses of *Laurencia* species CLs (n = 60)

Species	Lobes	
	Diameter (µm)	Length (µm)
Yuzurua poiteaui var.	0.47	4.52
SD	0.10	1.0
L. dendroidea	0.47	6.6
SD	0.05	2.0
L. translucida	0.15[a]	2.3[a]
SD	0.05	0.2

SD, standard deviation.
[a]Significant when <0.01 (α = 5%) ANOVA / Tukey Test.

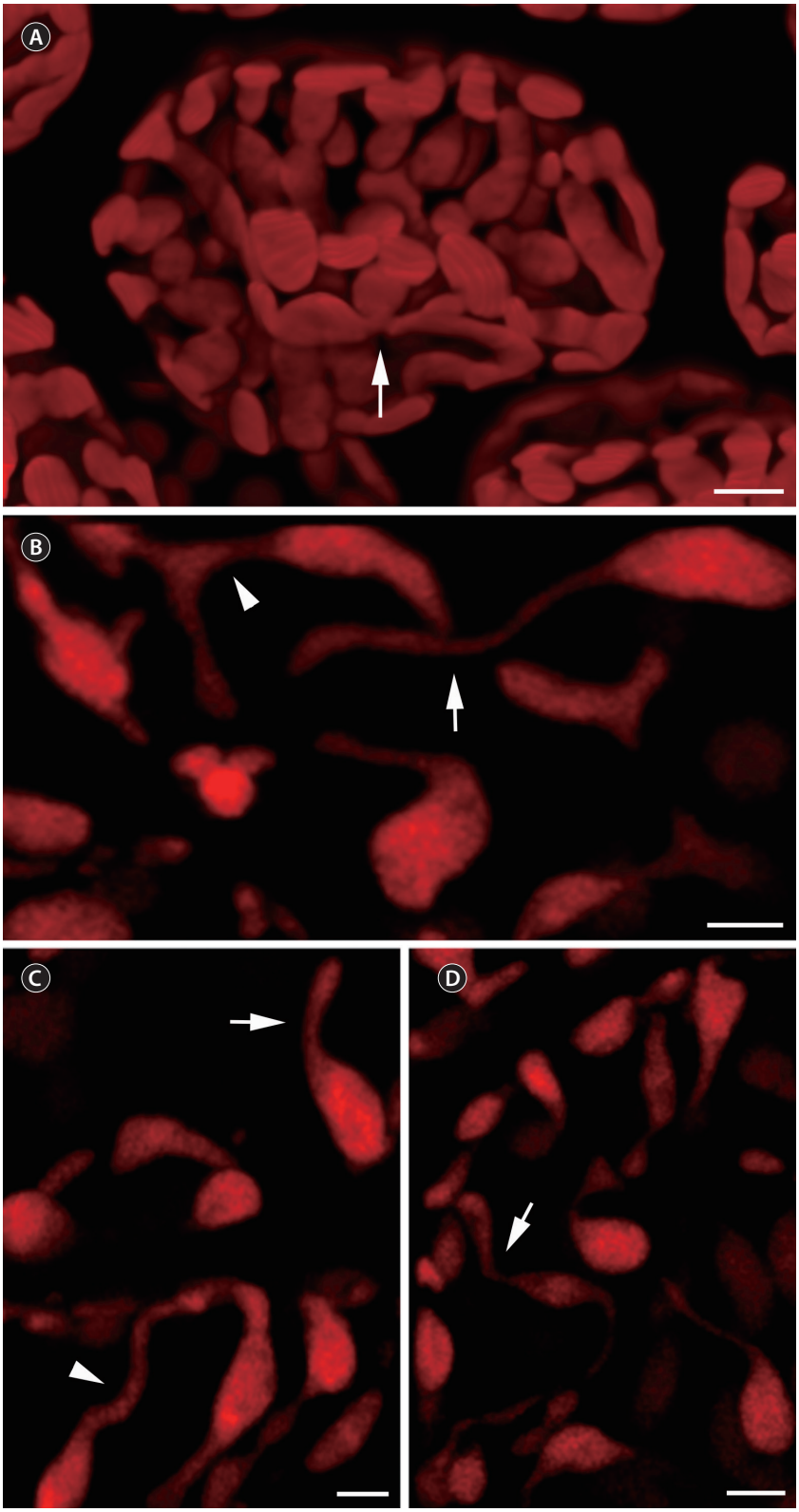

Fig. 2. Choroplast lobes (CLs) in *Laurencia*. (A) A three-dimensional reconstruction with confocal laser scanning microscopy (LSM) images of fusionned chloroplasts (arrow) in *Yuzurua poiteaui* var. cortical cells. (B) LSM images of thin undulated (arrow) and thick linear (arrowhead) CL in *L. dendroidea* cortical cells. (C & D) Short / unilateral (arrows) and thick / bilateral (arrowheads) CL in *L. dendroidea* cortical cells. Scale bars represent: A-D, 2 μm.

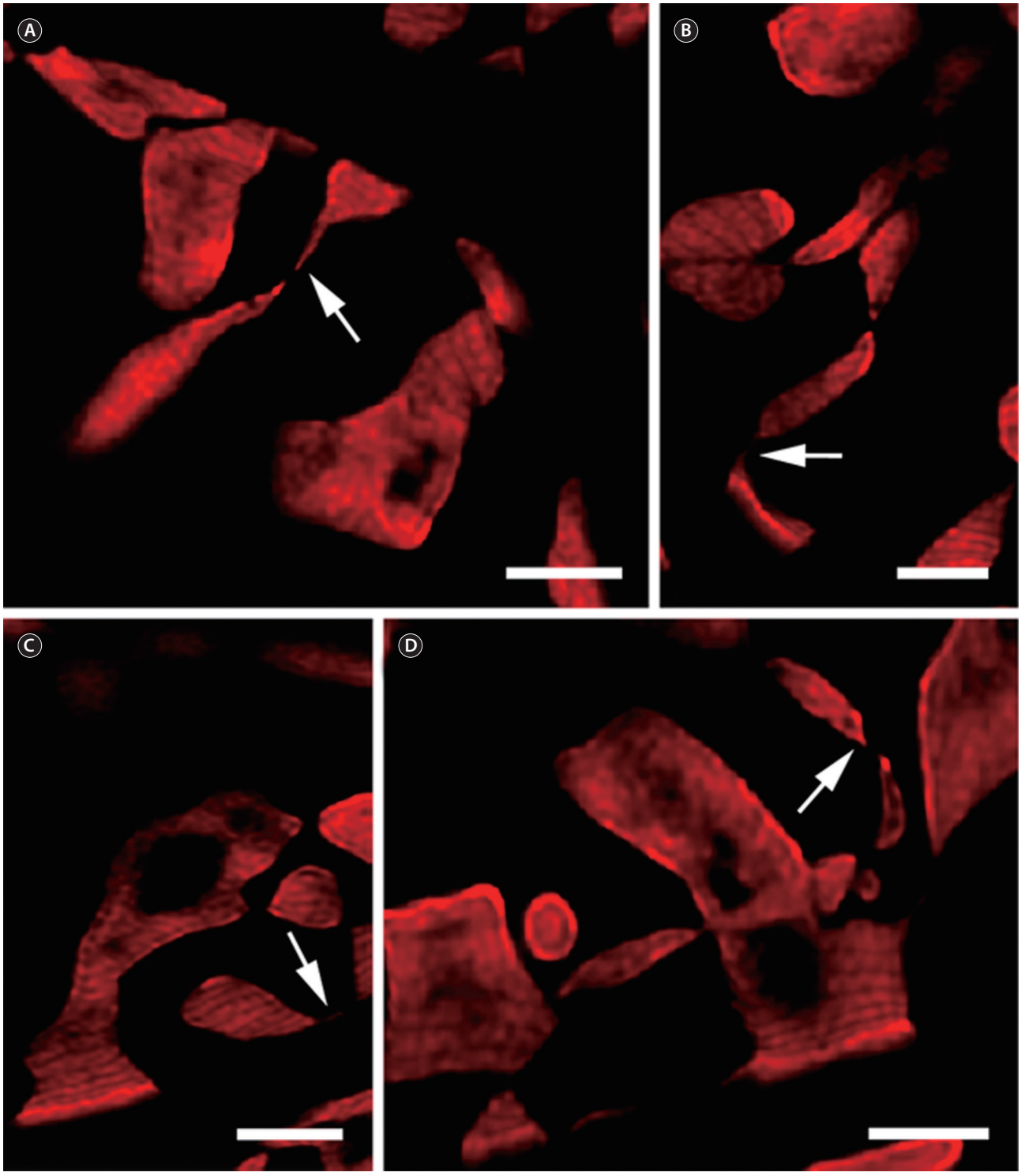

Fig. 3. Choroplast lobes (CLs) in *Laurencia translucida* cortical cells. (A & B) Confocal laser scanning microscopy images of thin / unilateral CLs (arrows). (C & D) CLs always as inconspicuous projections (arrows). Scale bars represent: A-D, 2 μm.

poiteaui var. CLs presented higher diameter and length than *L. translucida* CLs. Based on LSM observations, we suggest that lobes morphology do not have diagnostic value at the generic level. It can have a taxonomic validity only to characterize *L. translucida* species.

From all species of *Laurencia* complex, until now, only two of them were studied in cellular, ecological and chemical levels, *Laurencia dendroidea* (Da Gama et al. 2003, Sudatti et al. 2006, Salgado et al. 2008) and *Laurencia translucida* (Fujii et al. 2011, Paradas 2013). According to Garbary and Gabrielson (1990), the chemotaxonomy of red algae had been hampered by the strong intra-populational variability and by the production of secondary metabolites by thalli parasites or epibionts (Crews and Selover 1986, Da Gama et al. 2014), which in many cases difficult the taxonomic use of halogenated metabolites (Garbary and Gabrielson 1990).

But recently, morphological, molecular and chemical data from *Laurencia* species have been used to understand the evolution of this group (Fujii et al. 2011). For example, *Laurencia dendroidea* produces as major metabolites terpenoids with a broad scale of biological activity (e.g., antifouling) and stores it inside CC found in cortical cells (Salgado et al. 2008, Paradas et al. 2010), while *L. translucida* possess specialized translucent cortical cells, which are involved in fatty acids derivatives biosynthesis with biological activity (e.g., antifouling) (Paradas 2013). These works have been showing that specific secondary metabolites are biosynthesized inside algae cells (Salgado et al. 2008, Paradas 2013).

Regarding to CLs data, *L. translucida* and *Y. poiteaui* var. / *L. dendroidea* are examples of extremely different chloroplasts shapes. Future studies revealing the nature of *Laurencia* secondary metabolites allied to new morphological analyses (e.g., CLs or storage structures) should help the understanding of evolution and phylogenetic relationships in *Laurencia* complex.

In conclusion, this work described for the first time CLs in *Laurencia* species by using distinct cellular approaches; different chloroplasts morphologies were also observed among *Laurencia* species, which indicates for a possible taxonomic approach based on morphology of CLs.

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