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Mixed evolutionary traits of *Tolypella* (section Rothia, Charales) compared with *Chara* and *Nitella* shown by ultrastructure of vegetative internodal cells

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ABSTRACT

The ultrastructure of vegetative internodal cells in a representative of the genus *Tolypella* (section Rothia) is described for the first time for *Tolypella intricata*. The most striking difference compared with the cells of *Chara* and *Nitella* is that the four-layered lateral cell wall shows the regular presence of unique refractive lenticular thickenings, which we designate as "tolysomes", considering their unique ultrastructure elucidated here for the first time. Tolysomes greatly amplify the plasma membrane surface; thereby defining particular spaces of the cytoplasm. There are similar structures in the cell walls of the non-charalean charophyte *Coleochaete orbicularis* and also in bryophytes, angiosperms and gymnosperms. As the function of tolysomes is hitherto elusive, a bona fide analysis of evolutionary significance is not yet possible. C-shaped amitotic nuclei are similar to those described for young internodal *Chara* cells. Resting amitotic nuclei exhibit normal nuclear envelope, dispersed granular chromatin and numerous nucleoli with variable size. Nevertheless, they show neither the single, central nucleolus characteristic of dividing *Chara* nuclei nor the bundles of tubular elements running parallel to the long axis of the nucleus, which are also normal in *Chara* and *Nitella* amitotic nuclei. The presence of C-shaped resting nuclei of *Tolypella* and *Chara* and their absence in *Nitella* and the presence of spindle-shaped nuclei in *Nitella*, but not in *Tolypella*, suggest that *Tolypella* (section Rothia) divergence took place earlier than did *Nitella* in accordance with, but also in contradiction to molecular data. Mature echinoid bodies and chloroplasts in *Tolypella intricata* are entirely similar to those previously described for *Chara* and *Nitella*.

1. Introduction

The phylum Streptophyta (Bremer, 1985; Bremer et al., 1987) comprises land plants (liverworts, hornworts, mosses, ferns, conifers and flowering plants) and Charophycean green algae, whereas all other green algae belong to the lineage Chlorophyta (chlorophycean, oedogoniophycean, ulvophycean, trentepohliophycean, prasinophycean) (Lewis and McCourt, 2004). Six monophyletic orders of charophycean green algae are currently recognized: Mesostigmatales, Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales where the trend is the increase in morphological complexity (Graham and Wilcox, 2000).

Multigene analysis (Lewis and McCourt, 2004) and actin phylogeny (Bhattacharya et al., 1998) suggest the genus *Mesostigma* is in the base on divergence of the charophycean algae although the initial steps of the ontogeny of the flagellar apparatus of *Chara* suggest it is not a *Mesostigma*-like, but a *Mamiella*-like ancestor for Charales (Vouilloud et al., 2005, 2012).

With respect to the phylogenetic relationships among Charales and the other charophycean orders, Chapman and Waters' (2002) assertion for molecular studies that states that "every conceivable topography has been supported in the literature" is still valid. Nevertheless, it is consistent with the majority of studies (Surek et al.1994; McCourt, 1995; Friedl, 1997; Huss and Kranz, 1997; Nakayama et al., 1998; Marin and Melkonian, 1999; Karol et al., 2001; Qiu et al., 2006; Becker and Marin, 2009; Leliaert et al., 2012; Turmel et al., 2013; Rice et al., 2013; Zhong et al., 2014) that the Charales normally forms clades clearly separated from the other charophycean orders. Cytologically differences are clear since never appear organelle and structures in the rest of the charophycean orders that remain exclusive to Charales, such as amitotic nuclei, equinoid bodies, glycosomes, chloroplast granum-like structures, *etc*. with particular morphologies and ultrastructures (Pickett-Heaps, 1975; Cáceres and Cocucci, 1975; Franceschi and Lucas,

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1981a; Grant and Borowitzka, 1984). In this sense, ultrastructural developmental stages of the flagellar apparati of male gametes of *Chara* (Pickett-Heaps, 1968; Moestrup, 1970; Cocucci and Cáceres, 1976; Duncan et al., 1997; Vouilloud et al., 2005, 2012) and *Nitella* (Turner, 1968; Vouilloud et al., 2010) present radical differences from the ontogenies of those of non-Charalean charophytes' gametes, such as in Coleochaetales (Graham and McBride, 1979; Graham and Wedemayer, 1984; Graham and Repavich, 1989) (see Vouilloud et al., 2005).

There is no consensus about which of the charophycean orders gave rise to the land plants. Gontcharov (2008) and Leliaert et al. (2012) claim that there is still uncertainty concerning the precise relationships between land plants and their green algal relatives. Since the study of Karol et al. (2001), there has been no doubt about the fact that embryophytes emerged from a charophycean genus. The lack of consensus of molecular studies mainly focuses on elucidating whether Zygnematales, Coleochaetales, Zygnematales plus Coleochaetales or Charales alone are the orders sister to land plants (Surek et al., 1994; Karol et al., 2001; Delwiche et al., 2002; Turmel et al., 2006, 2013; Becker and Marin, 2009; Leliaert et al., 2012; Laurin-Lemay et al., 2012; Zhong et al., 2013, 2014). In this sense Turmel et al. (2013) recommend caution when large multigene sequence alignments with a limited taxon sampling are analyzed. Recently, Rice et al. (2013) indicated horizontal transfer of entire mitochondrial genomes from three green algae (including *Chlorokybus* and *Chara*) in one of the most ancient flowering plants.

Molecular methods of dating point to an early origin of embryophytes (Graham et al., 2012). Sanderson (2003) considered the land plant split to be Ordovician, a date that is supported by the fossil spore record (Wellman et al., 2003). However, more recent studies suggest a Precambrian origin for the embryophytes (Clarke et al., 2011).

Tolypella and *Nitella* are presently considered genera members of the tribe Nitellae by possessing oogonia with small coronula with 10 cells in two tiers (Groves and Bullock-Webster, 1920; Wood and Imahori, 1965; Cáceres, 1978). Nevertheless, *Tolypella* also shares characteristics with the tribe Chareae since it has oogonia and oospores circular in cross section and monopodial branchlets with lateral antheridia (see Cáceres, 1978). At the molecular level (McCourt et al., 1996, 1999; Sakayama et al., 2002) *Tolypella* and *Nitella* occupy different clades.

Wood and Imahori (1965) frankly admitted inability to provide convincing evidence to show that *Tolypella* was a distinct genus from *Nitella* and more exactly it could easily be constructed as a section of that genus. Sawa (1974), on the contrary, concluded that with exception of the 10-celled coronula, the genus *Tolypella* shares certain important morphological and anatomical features with the tribe Chareae, rather than with the genus *Nitella*. Thus he considered that it is quite likely on cytological as well as on morphological and anatomical grounds that the three taxonomic groups in Charales, the tribe Chareae and the genera *Tolypella* and *Nitella* have diverged by aneuploidization, and he also stated that the phylogenetic distance between *Tolypella* and *Nitella* is not much closer than that between *Tolypella* and Chareae.

Similarly, both of the basically different oogonial structures: Chareae-type, where the oosphere is accompanied by a single sterile cell, and *Nitella*-type, where the oosphere has three sterile cells (Sawa and Frame, 1974) are present in *Tolypella*. This fact especially suggests that the genus *Tolypella* may occupy a phylogenetic position between the tribe Chareae and the genus *Nitella*. More specifically, Sawa and Frame (1974) concluded that since plants belonging to section Rothia Wood 1962 (*sensu* Wood and Imahori, 1965) possess the Chareae-type oogonial structure and those of section Tolypella Wood 1962 (*sensu* Wood and Imahori, 1965) are distinguished by the *Nitella*-type oogonium, the traditional subdivision of the genus into the two sections based on whether the branchlet end cell is acute or obtuse is further substantiated by the difference in oogonial structure. Both sections are also separated at molecular level (McCourt et al., 1996).

Soulié-Märsche and García (2014) consider that although the oospores of the two sections of *Tolypella* are overall similar, the difference in the number of the sterile oogonial cells is so significant that the sections are not compatible with their belonging to the same genus. Moreover, as the gyrogonites produced by the extant species of section Rothia Wood 1962 (*sensu* Wood and Imahori, 1965) are identical to the gyrogonites of the fossil genus *Sphaerochara* Mädler 1955, Soulié-Märsche (1989), according to the rules of botanical nomenclature, transferred the living species of *Tolypella* with acute branches to this genus. Thus the genus *Tolypella* A. Braun was amended and limited to species with multiple sister cells by this author.

We hypothesized that all these generic morphological and cytological distinctions could be reflected in the ultrastructure of *Tolypella* vegetative cells. McCourt et al. (1996) and Duncan et al. (1997) stressed the need to obtain precise data on the morphology of extant and fossil charophytes in order to complement molecular studies to elucidate the relationships among the charophycean green algae and land plants. Chapman and Waters (2002) also stressed the necessity of noticing the importance of morphological characters in resolving the question of land plant ancestry. Graham et al. (2012) also emphasized the relevance of ultrastructural information suggesting that ultrastructural traits of modern *Coleochaete* may provide models to be compared with images of fossil Streptophyta.

Since no ultrastructural studies have been made for any representative of the genus *Tolypella*, the principal goal of the present study is to elucidate the ultrastructure of vegetative internodal cells of *Tolypella intricata* (sect. Rothia). The descriptions of the ultrastructure of vegetative cells in Charales have been conducted for several species of genus *Chara* (Chambers and Mercer, 1964; Barton, 1965a; Pickett-Heaps, 1967; Ducreux, 1968; Cáceres and Cocucci, 1975; Franceschi and Lucas, 1980, 1981a,b; Stabenau et al., 2003). Some studies have also been made in species of *Nitella*, but focused on particular organelles (Green, 1959, 1964; Silverberg and Sawa, 1973; Allen, 1974). The cell wall has widely been studied in both young and mature cells of *Chara* (Fridvalszky and Lovas, 1958; Barton, 1968; Ducreux, 1968; Pickett-Heaps, 1967; Fischer et al., 1974; Franceschi and Lucas, 1981a) and *Nitella* (Green, 1954, 1958, 1959; Green and Chapman, 1955; Probine and Preston, 1958, 1961; Spanswick and Costerton, 1967; Allen, 1980; Neville and Levy, 1984).

The ultrastructure of the vegetative cells of Charales is well known on the basis of the aforementioned studies in *Chara* and *Nitella*. A large, central, vacuolar system occupies most of the cell volume and a thin layer of protoplast is located against the cellulose cell wall. Aligned chloroplasts (Pickett-Heaps, 1967; Kamitsubo, 1972) and huge amitotic nuclei (Vouilloud et al., 2007) are present. Chloroplasts showed granum-like structures (Pickett-Heaps, 1967; Cáceres and Cocucci, 1975). Amitosis has been studied in species of *Nitella* and *Chara*, (Cáceres and Parodi, 1985; Parodi and Cáceres, 1991; Vouilloud et al., 2007) but also in *Nitellopsis* (Foissner and Wasteneys, 2000). Other conspicuous cytoplasm inclusions are echinoid bodies, which are refractile irregular, spherical structures of 20–40 μm in diameter (Pickett-Heaps, 1975). Echinoid bodies were first described in the nineteenth century (Overton, 1890) and named years later as "protein inclusions" or "spheres hair" (Votava, 1914; Groves and Bullock-Webster, 1920). Costerton and Robbie (1970) were the first to observe them with transmission electron microscopy (TEM) in *Nitella translucens*, and Silverberg and Sawa (1974a) studied these inclusions in *Nitella flexilis* with TEM and light microscopy. Cyclosis is associated with actin filaments (Kamitsubo, 1972; Williamson, 1993) and it is easily perceptible

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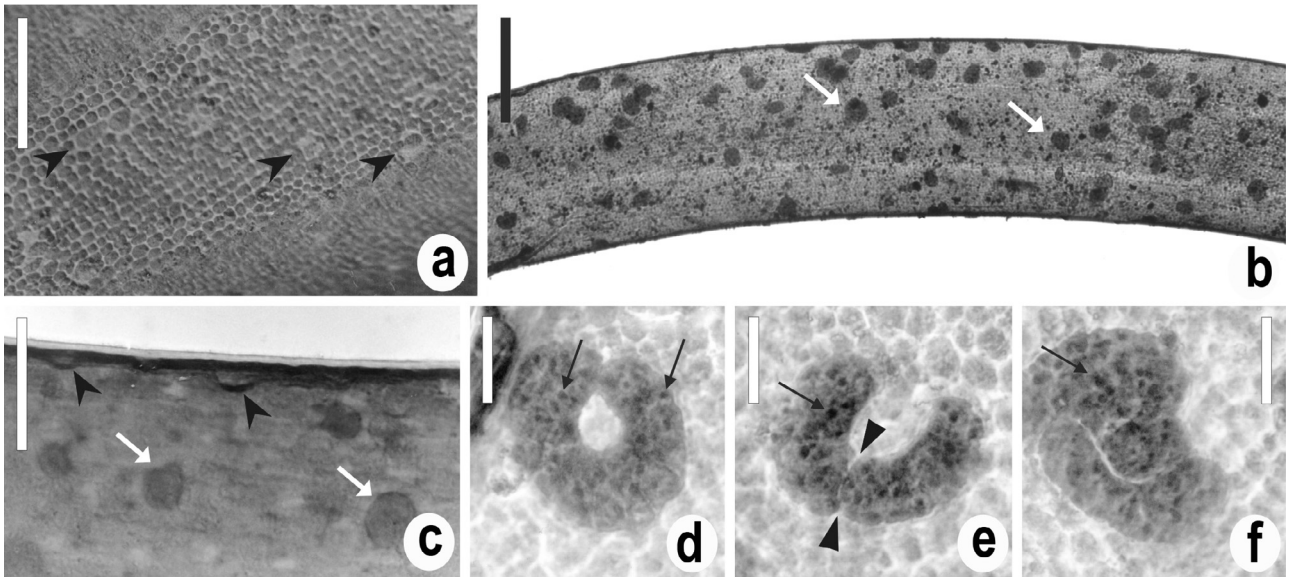

Fig. 1. *T. intricata*. Light microscope images of vegetative internodal cells. (a) Frontal view of a living cell showing the layer of aligned chloroplasts and tolysomes (arrowheads). Scale = 50 μm. (b) Young axial, internodal cell of 3 mm in length; numerous amitotic nuclei are visible (arrows). Scale = 200 μm. (c) Detailed view of (b) showing the thick, stratified cell wall. Tolysomes are seen laterally (dark arrowheads). It is clear that they are the result or modifications of the wall's internal strata. White arrows pinpoint amitotic nuclei. Scale = 50 μm. (d–f) Details of amitotic nuclei. (d) Resting C-shaped amitotic nucleus; arrows signal nucleoli stained with propiocarmine. Scale = 20 μm. (e) Recently divided amitotic nuclei; the arrowheads indicate the plane of division. The arrow pinpoints a nucleolus. Scale = 20 μm. (f) A group of recently divided amitotic nuclei, which have remained close together after division. Scale = 20 μm.

in internodal cells by the apparent motion of nuclei and echinoid bodies. Membrane-bound inclusions called glycosomes are a common feature of both young and mature cells of *Chara* (Franceschi and Lucas, 1981b).

2. Methods

T. intricata (Trent. ex Roth.) Leonh. (see Cáceres, 1978) was collected in a flood lagoon of Paraná city, Entre Ríos Province, Argentina. For *in vivo* observation of cell wall structures ("tolysomes"), a 0.5%, pH 5 aqueous solution of Alcian Blue (AB) (Parker and Diboll, 1966) was used as stain. It was dripped onto the material, which was mounted in water on a glass slide. For light microscopy observations of vegetative amitotic nuclei, the material was fixed in Carnoy I and stained with propiocarmine (0.5% carmine solution in propionic acid 45%), according to Wells and Hoshaw (1971).

For TEM, thalli portions were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer for 2 h and postfixed in 2% osmium tetroxide in 0.05 M cacodylate buffer for 2 h. Then, the material was successively dehydrated in a series of progressively more concentrated rinses of acetone (20–100%) and embedded in Spurr resin. Ultrathin diamond knife sections were contrasted with uranyl acetate/lead citrate and observed under a JEOL electron microscope (Jeol Ltd., Tokyo, Japan) at the CCT-CONICET, Bahía Blanca.

3. Results

3.1. Light microscopy

Lateral cell walls of mature internodal cells exhibit numerous refractive and Alcian Blue positive thickenings, circular and with rough contour in frontal views, which we call "tolysomes" (Fig. 1a–c). Chloroplasts are linearly arranged (Fig. 1a) and cells display numerous amitotic nuclei (Fig. 1a–f). The resting amitotic nuclei are C-shaped (Fig. 1d), and contain homogeneous granular chromatin and numerous small, spherical, ovoid or fusiform scattered nucleoli. During amitosis, nuclei are similar in shape, size and content to the resting ones (Fig. 1e–f). Groups of 4 or more recently divided nuclei are commonly present (Fig. 1f).

3.2. Electron microscopy

The cell wall is over 5 μm thick in mature cells and shows four layers (Fig. 2a). These comprise an electron-dense, thin (about 0.2 μm wide), outermost layer (Fig. 2a and e) and three other readily distinguishable layers: one fibrillar, 2.5 μm wide, homogeneous and with clear crystalline appearance (Fig. 2a and f); another one thin, spongy, about 0.3 μm wide (Fig. 2a and m) and the innermost one with variable thickness (between 1.5 and 2.5 μm), fibrillar, with median electron-density (Fig. 2a and i). Tolysomes (Fig. 2b–e) are the result of dome-shaped thickenings of the thin spongy layer, which causes both fibrillar layers to separate from each other (Fig. 2b–c). In turn, the innermost layer produces numerous internal, irregular lobulations that plasmalemma covers entirely (Fig. 2d–e) forming a complex labyrinth of tubules. Toward the cytoplasmic side of tolysomes, associated mitochondria are commonly observed, in some cases in contact with the folded plasmalemma (Fig. 2d, arrow). Putative glycosomes are also in association with tolysomes (Fig. 2d and g).

The cells show a large central vacuole that occupies most of the cell volume (Fig. 2f–g). The chloroplasts are aligned with their long axes parallel to the longitudinal axis of the cell (Fig. 2f–g and c) displaying multitylakoid lamellae (Fig. 2a and f–g), commonly organized in grana (Fig. 2a), starch granules and lipid globules (Fig. 2g, s and l).

Amitotic nuclei have a normal nuclear envelope, dispersed granular chromatin and numerous nucleoli with variable size (Fig. 2h). Spherical structures with high electron density and clear center are common (Fig. 2h, arrow).

Echinoid bodies (Fig. 2i and j) are roughly spherical, up to 15 μm in diameter, with irregular projections (Fig. 2i). They consist of a homogeneous core with medium electron opacity (Fig. 2j, co), limited by a thin, electron-dense layer (Fig. 2j, cl). They are always in close association with a membrane (Fig. 2j, arrowheads).

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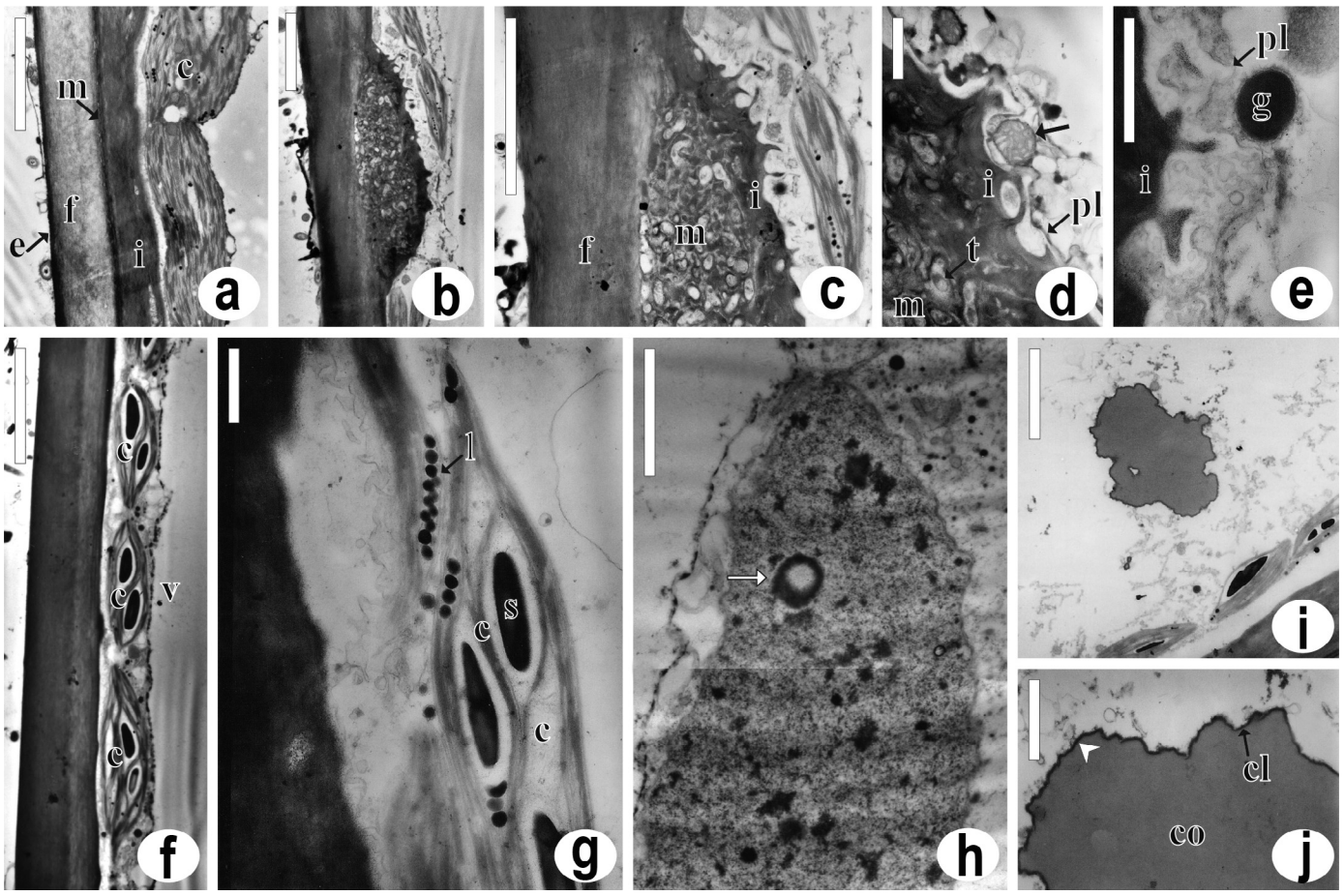

Fig. 2. *T. intricata*. TEM images of vegetative internodal cells. (a) Detail of the cell wall composed of four layers, an electron dense, thin, outermost layer (e) and three other readily distinguishable layers: one fibrillar, homogeneous and with clear crystalline appearance (f); another one, thin, spongy (m) and the innermost fibrillar and median electron dense (i). Scale = 5 μm. (b) Tolysome lengthwise sectioned showing shape and appearance. Scale = 5 μm. (c) Detail of (b) to show the tolysome structure. Note that the spongy layer inside the tolysome gradually separates the fibrillar layers (f) and (i), and that the internal surface of the inner layer is extremely irregular. Scale = 5 μm. (d) Detail of the tolysome internal surface adjacent to the protoplast. Note the plasmalemma (pl) covers all finger-like projections of the internal layer of the wall (i). A tubule (t) of the layer (m) is pinpointed. A mitochondrium profile is seen associated with the plasmalemma (arrow). Scale = 1 μm. (e) Detail of the cytoplasm of a cell showing a putative glycosome (g) in association with the tolysome. Scale = 1 μm. (f) Detail of a cell showing a portion of the large central vacuole (v) that occupies most of the cell volume and the layer of discoid chloroplasts (c) aligned with their long axes parallel to the longitudinal axis of the cell. Scale = 5 μm. (g) Detail of a chloroplast (c); it shows multitylakoid lamellae, starch granules (s) and lipid globules (l). Scale = 1 μm. (h) Longitudinal section of an amitotic nucleus. Note numerous nucleoli and uniform chromatin. The arrow indicates a spherical inclusion with high electron density and clear center. Scale = 3 μm. (i) Echinoid body. Scale = 5 μm. (j) Detail of an echinoid body. It shows a central core (co) with medium electronic opacity and an outer, thin electron dense layer (cl) associated with membrane elements (arrowhead). Scale = 2 μm.

4. Discussion

The ultrastructure of vegetative, internodal cells of *T. intricata* shows similarities to and differences from those of both *Chara* and *Nitella* (*sensu* Groves and Bullock-Webster, 1920; Wood and Imahori, 1965). The most striking peculiarity is that the cell wall shows the regular presence of unique refractive lenticular thickenings, which we have named as "tolysomes", considering their ultrastructure elucidated here for the first time, since previous studies of these structures are light microscopy works. Fridvalszky (1958) visualized them with special techniques in *T. intricata* and called them "pits". Sawa and Markoff (1982) indicated that the presence of the pitted wall structures was a distinct taxonomic feature specific to the genus *Tolypella* in comparison with the other major genera of Charales, *i.e. Chara*, *Nitella* and *Lamprothamnium*. Langangen (1994) also observed these structures in *T. normaniana* and *T. nidifica* and called them "callose pits". The present ultrastructural study shows that tolysomes occur as dome-shaped thickenings of the thin spongy layer of the cell wall, which cause a separation between the fibrillar layers. It also shows clearly that the tolysomes define particular cytoplasm spaces owing to a great enlargement of the plasma membrane surface.

There are also elaborate, localized cell wall ingrowths in the cell walls of the non-Charalean charophyte *Coleochaete orbicularis*, present in cells adjacent to developing zygotes during the stage of reserve material accumulation (Graham and Wilcox, 1983; Graham and Kaneko, 1991). Similar structures have also been found in placental transfer cells of bryophytes, where labyrinth cell walls are well developed in cells of the gametophyte in contact with the sporophyte, where the transfer of solutes from gametophyte to sporophyte is documented (Kelley, 1969; Browning and Gunning, 1979; Thomas et al., 1979). Labyrinth wall projections have also been widely described for transfer cells in angiosperms and gymnosperms (Pate and Gunning, 1972; Schmidt and Bartels, 1996). As the function of tolysomes has proven hitherto elusive, a bona fide analysis of its evolutionary significance is impossible at present. Nevertheless, in all cases homologies could be ruled out since all the structures are located in walls of contiguous cells, but not placed in lateral walls in contact with the environment as tolysomes.

In the genus *Chara*, there is a different kind of plasmalemma modification called a charasome (Barton, 1965b; Franceschi and Lucas, 1980; Lucas and Franceschi, 1981; Graham and Kaneko, 1991). Charasomes are produced by the anastomosis of tubules derived from plasmalemma but they are not coupled with the

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cell wall modifications present in tolysomes, so it would not be reasonable to consider tolysomes and charasomes homologous structures. Eventually, knowledge of the function of tolysomes will provide data to decide the morphological and functional homology or homoplasy of both structures.

The internodal cells of *T. intricata* shows a single central vacuole similar to that in *Chara* cells (Pickett-Heaps, 1975) but different from the central multivacuole system of *Nitella* cells (Silverberg and Sawa, 1974b). Echinoid bodies (Pickett-Heaps, 1975; Silverberg and Sawa, 1974a; Homblé and Foissner, 1993) and chloroplasts (Pickett-Heaps, 1975; Cáceres and Cocucci, 1975) are entirely similar to those described previously in *Chara* and *Nitella.*

The C-shaped amitotic nuclei of *T. intricata* are very similar to those described for the young central internodal cells of *Chara corallina* (Shen, 1967) and *C. contraria* (Vouilloud et al., 2007), but different from the spindle-shaped nuclei of *Nitella hyalina* (Cáceres and Parodi, 1985; Parodi and Cáceres, 1991) and *C. contraria* (Vouilloud et al., 2007). Nevertheless, dividing nuclei in *T. intricata* do not show a single, central nucleolus characteristic of dividing nuclei of *C. contraria* (Vouilloud et al., 2007), but exhibit many and small nucleoli as in the dividing nuclei of *N. hyalina* (Parodi and Cáceres, 1991). Moreover, the dividing nuclei of *T. intricata* do not show the bundles of tubular elements running parallel to the long axis of the nucleus that are normal in the nuclei of *Nitella* (Parodi and Cáceres, 1991) and *Chara* (Pickett-Heaps, 1967; Barton, 1967; Foissner and Wasteneys, 2000; Vouilloud et al., 2007). In comparing the amitotic nuclei of *Tolypella* with those of *Chara* and *Nitella*, their similarities and differences are relevant since traits of nuclear divisions have been considered significant in speculations of algal taxonomy (Pickett-Heaps, 1975; Mattox and Stewart, 1984). The following features are remarkable: (1) C-shaped resting nuclei appear in *Tolypella* and *Chara* but never in *Nitella*, (2) spindle-shaped resting nuclei appear in *Nitella* and *Chara* but never in *Tolypella*, (3) the single central nucleolus typical of dividing nuclei of *Chara* never appears in *Nitella* and *Tolypella* and, (4) the bundles of longitudinal tubular elements of dividing nuclei clearly apparent in *Nitella* and *Chara* are absent in *Tolypella.* In particular, the presence of C-shaped resting nuclei of *Tolypella* and *Chara* and the absence of spindle shaped nuclei in *Tolypella* support the divergence of *Tolypella* being prior to the divergence of *Nitella*, in agreement with McCourt et al. (1996, 1999), Graham and Wilcox (2000) and Sakayama et al. (2002), but also contradicting other molecular results by Meiers et al. (1997) and Sanders et al. (2003). It could be speculated that the spindle-shaped amitotic nuclei in *Nitella* (Parodi and Cáceres, 1991) and the single central nucleolus in dividing nuclei of *Chara* (Vouilloud et al., 2007) are apomorphic traits, but considering that the present study resulted from an evaluation of only a single species of *Tolypella*, section Rothia, all taxonomic/phylogenetic conclusion are premature when there are 20 currently accepted species of the genus (see Guiry and Guiry, 2014). Furthermore, the genus *Tolypella* is clearly morphologically divided in two sections, Rothia and Tolypella (*sensu* Wood and Imahori, 1965), both also separated at molecular (McCourt et al., 1996), anatomical (Sawa and Frame, 1974) and cytological (Sawa, 1974) levels.

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