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Host–parasite relationship of the geoduck *Panopea abbreviata* and the green alga *Coccomyxa parasitica* in the Argentinean Patagonian coast

Nuria Vázquez [a,*], Francisco Rodríguez [b], Cristián Ituarte [c], Javier Klaich [a], Florencia Cremonte [a]

[a] Centro Nacional Patagónico (CONICET), Boulevard Brown 2915, U9120ACD Puerto Madryn, Chubut, Argentina
[b] Instituto Español de Oceanografía, Apdo. 1552, 36200 Vigo, España, Spain
[c] Museo Argentino de Ciencias Naturales (CONICET), Av. Ángel Gallardo 470, C1405DJR Buenos Aires, Argentina

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ABSTRACT

The association of the geoduck *Panopea abbreviata* and the green alga *Coccomyxa parasitica* is described. The identity of the green alga was confirmed by molecular studies; the alga was found within the hemocytes that infiltrate the connective tissue of the geoduck siphons. Cytological characteristics of hemocytes were not altered by algal infection; very often the algae were seen enveloped by a digestive vacuole within the hemocyte cytoplasm, evidencing diverse degrees of resorption. Connective cells of siphons were rarely infected by *C. parasitica*. The mean prevalence of *C. parasitica* was higher (82%) in San Matías Gulf (42°00′S, 65°05′W) than in San José Gulf (45%) (40°32′S, 64°02′W); except for spring, when the two locations showed no differences in prevalences (80%). Independently of location, season and host size, infected geoducks showed lower condition index values than uninfected ones. Regarding other bivalve species, only one specimen of the razor clam *Ensis macha* was found infected, and none of the oysters *Ostrea puelchana* and *Pododesmus rudis* and scallop *Aequipecten tehuelchus* was parasitized by the green alga.

1. Introduction

The geoduck *Panopea abbreviata* (Valenciennes, 1839) (Hiatellidae) is endemic from the Southwestern Atlantic, occurring between 23°S and 48°S (Scarabino, 1977). This species is a large and long-lived bivalve which is found deeply buried, up to 70 cm in soft muddy sediments; only the tips of the siphons remain exposed above the sediment surface (Morsan and Ciocco, 2004). Nevertheless, this species represents a valuable potential fishery resource; the exploitation of their Atlantic populations started only in the recent years (Ciocco, 2000).

During a survey of the health status of *P. abbreviata* in Northern Patagonian gulfs (Argentina), some geoducks showed the tips of siphons to be green colored, resembling infections by the intracellular green algae of the genus *Coccomyxa* (Chlorococcales: Coccomyxaceae) described by Rodríguez et al. (2008) in *Mytilus edulis*.

The genus *Coccomyxa* includes both free-living marine and freshwater planktonic species (Guiry et al., 2005), epiphytic (Lamenti et al., 2000), symbiotic with lichens (Lohtander et al., 2003) and protozoans (Hoshina and Imamura, 2008), and parasitic in starfish (Mortensen and Rosenvinge, 1933), scallops (Naidu and South, 1970; Stevenson and South, 1974), and mussels (Boraso de Zaixso and Zaixso, 1979; Bala, 1995; Gray et al., 1999). *C. parasitica* was first described by Stevenson and South (1974), infecting the giant scallop *Placopecten magellanicus* (Pectinidae) from Newfoundland, Canada. It was also reported in *M. edulis chilensis* from Patagonia, Argentina (Boraso de Zaixso and Zaixso, 1979; Bala, 1995) and from Malvinas (Falkland) Islands (Gray et al., 1999). However, the identification of the alga in the last mentioned studies was only supported by morphological features. Rodríguez et al. (2008) confirmed the presence of *C. parasitica* infecting *M. edulis chilensis* in the North Sea and Malvinas (Falkland) Islands, based on data from molecular studies, histopathology, ultrastructure, and pigments of the green alga.

In the present study, the presence of *C. parasitica* in *P. abbreviata* from Northern Patagonian gulfs is reported for the first time, and the alga was characterized based on both morphological and molecular data (small subunit ribosomal RNA (SSU rRNA) sequencing). Furthermore, the seasonal and geographical variations of prevalence and the effects of the green alga on the condition index of geoducks were studied.

2. Materials and methods

2.1. Sample collection and processing

During 2007, 60 geoducks (*P. abbreviata*) were seasonally collected at 15 m depth at Puerto Lobos (42°00′S, 65°05′W), San

* Corresponding author. Fax: +54 2965 451543.
E-mail address: nuria@cenpat.edu.ar (N. Vázquez).

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Matías Gulf ($n = 240$), and approximately 50 geoducks at Punta Conos (40°32′S, 64°02′W), San José Gulf ($n = 210$) (Fig. 1). Each sampling season was carried out in January, April, July and October for both locations. Additionally, during 2006 and 2007, 682 oysters [*Ostrea puelchana* d'Orbigny, 1842 (Ostreidae)], 133 jingle-shell oysters [*Pododesmus rudis* Broderip, 1834 (Anomiidae)] were collected at Puerto Lobos and Playa Fracasso (42°25′S, 64°07′W), 480 razor clams [*Ensis macha* (Molina, 1782) (Solenidae)] at Playa Fracasso and 180 scallops [*Aequipecten tehuelchus* (d'Orbigny, 1846) (Pectinidae)] at Punta Conos (Fig. 1). The bivalves were transported to the laboratory and maintained in aquaria with aerated seawater at 13 °C for 24 h until processing.

Maximum shell length of each specimen was measured; shell and flesh were weighed separately to calculate the condition index (wet flesh weight to shell weight ratio). Soft parts of all bivalves were macroscopically examined for signs of algal infection, as the extent of green color, presumed to indicate algal colonization or infection. Infection intensity, based on the extension of the green area on the siphons, was graded as: uninfected: absence of green coloration; slightly infected: green color confined up to 1 cm from the tip of the siphons; moderately infected: green color reaching up to 3 cm from the tip of the siphon; heavily infected: a dark green color exceeding the 3 cm from the tip of the siphons.

2.2. Histological processing

Small pieces of siphonal tissues showing green coloration as well as pieces of nondiscolored siphonal tips (5 × 10 mm) were obtained, and one oblique transverse 5 mm thick section of 180 geoducks, containing gill, digestive gland, mantle, nephridia and gonad was excised. The siphonal tissues and the oblique transverse sections were fixed in Davidson's fixative (Shaw and Battle, 1957), dehydrated in an ethanol series and embedded in Paraplast® and Historesin® Leica. Histological sections (5 μm thick) were stained either with Harris or Mayer's hematoxylin and eosin, and observed under a light microscope.

2.3. Transmission electron microscopy

Small pieces of siphonal tissues showing green coloration (5 × 10 mm) were fixed in cold 2.5% glutaraldehyde with 4% formalin (from paraformaldehyde) in 0.2 M cacodylate buffer at pH 7.2 for 1 h. After rinsing in cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer at 4 °C, rinsed in 0.2 M cacodylate buffer, dehydrated in an ascending ethanol series (70–100%), and transferred to Spurr's resin via propylene oxide. Infiltration was performed in Spurr's resin. Ultrathin sections were double stained with uranyl acetate and lead citrate, and examined either in a Jeol 1200 EX II and a Philips EM301 transmission electron microscopes (TEM).

2.4. DNA extraction and SSU rRNA sequencing

Pieces of tissues from two geoducks showing green coloration were cut off and preserved in ethanol 96% until molecular analysis was performed. A green supernatant obtained after grinding tissues with a spatula was pipetted in an Eppendorf microtube and centrifuged (5000 rpm, 5 min) using a table-top minicentrifuge. A greenish pellet mixed with tissue debris was collected. The supernatant was centrifuged again (12,000 rpm, 10 min) and a second dark green pellet was obtained. Both pellets were rinsed in 100 μl of N-cetyl N,N,N,-trimethylammonium bromide (CTAB)

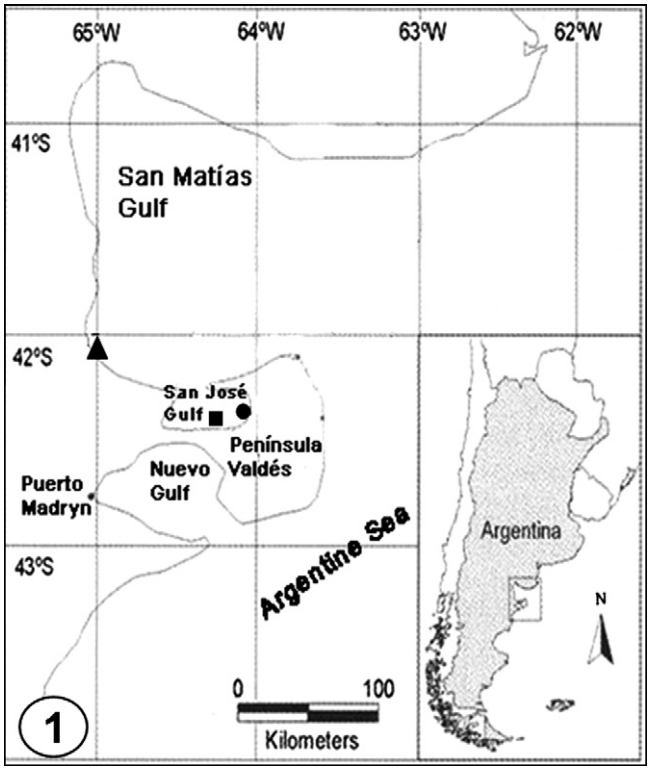

Fig. 1. Collection sites. References: ▲ = Puerto Lobos, ● = Punta Conos, ■ = Playa Fracasso.

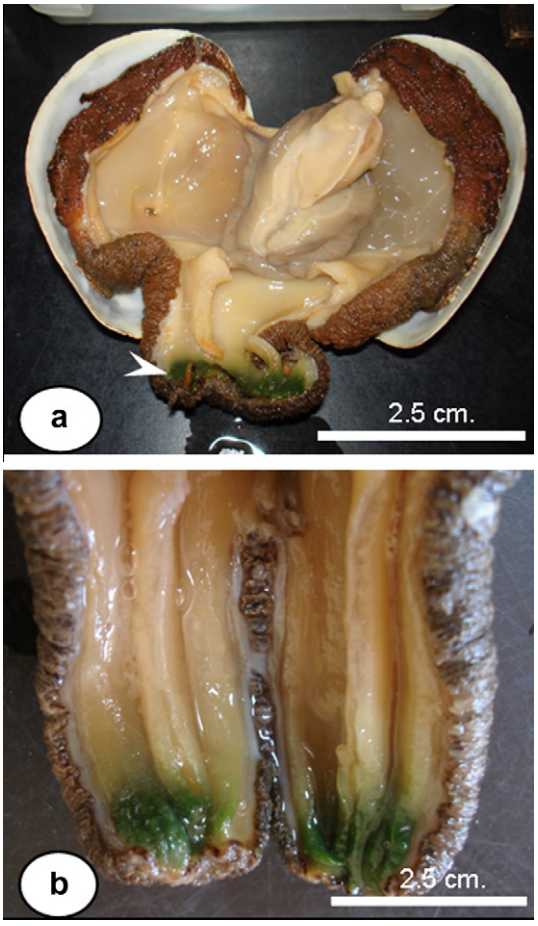

Fig. 2. *Panopea abbreviata*. Dissected clam (a) and detail of siphons (b) showing a moderate intensity of infection (arrow head).

extraction buffer (Ishida et al., 1999) and transferred to a 2 ml centrifuge tube. A quantity of glass beads (G-8772, Sigma Chemical Co., St. Louis, MO, USA) sufficient to fill the conical portion of the centrifuge tube was then added. A MiniBeadBeater (Biospec Products, Bartlesville, OK, USA) was then used to disrupt the cells, with agitation for 20 s at top speed. The mixture was then centrifuged at 5000 rpm for 5 min to separate the phases. The aqueous phase was removed to a new microcentrifuge tube and DNA extracted following a modified 1% N-cetyl N,N,N,-trimethylammonium bromide (CTAB) protocol (Ishida et al., 1999). The possibility of obtaining SSU rRNA sequences from the infecting algae using specific primers to avoid the simultaneous amplification of host SSU rRNA sequences and circumventing, if possible, the cloning step, was tested. A partial SSU rRNA gene sequence (568 bases, identical for the two pellets above mentioned) was amplified using the oligonucleotide primers (SSUF) 5′-CCG ACT CGC GGT GAA TCA-3′ and (SSUR) 5′-GGC CAG AGT CCT ATC GTG-3′. The primers were designed based on the SSU rRNA dataset used by Rodríguez et al. (2008) to amplify partial SSU rRNA sequences belonging to Trebouxiophyceae, including the *Coccomyxa*-like algae found in *M. edulis*. Additional SSU rRNA sequences from *Panopea japonica* and *P. abrupta* were inspected to confirm that they did not match SSUF and SSUR primers. The procedure for the PCR reaction was 1 min at 95 °C, followed by 34 cycles of 1 min at 95 °C, 2 min at 53 °C, 3 min at 72 °C, and a final extension of 7 min at 72 °C. SSU rRNA sequences were determined using the sequencing facilities at CACTI (University of Vigo, Spain). The obtained sequence of SSU rRNA was deposited in GenBank under accession number GU130257.

2.5. Phylogenetic analyses

The phylogenetic analysis included partial length SSU rRNA from other trebouxiophyceans deposited in GenBank for a total of 43 sequences in the final data set. Two out group sequences, *Trebouxia impressa* and *T. asymmetrica*, were used to root the trees. Partial SSU rRNA sequences (corresponding to sites 311–879 in the full 18S alignment) were aligned using CLUSTALW multiple alignment in BioEdit (Hall, 1999). Different nested models of DNA substitution and associated parameters were estimated using Modeltest 3.06 (Posada and Crandall, 1998). The Akaike information criterion (AIC) in Modeltest selected Tamura and Nei (1993) model with invariable sites and γ distribution (TrNef + I + G, proportion of invariable sites = 0.3990) and distribution of rates at variable sites with shape parameter (α) = 0.5071. The maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML) methods were used for the phylogenetic analysis (PAUP*4.0b10 version, Swofford, 2002) and bootstrap values were estimated from 1000 replicates.

2.6. Quantitative analysis

Differences in the prevalence of *C. parasitica* infection among geoducks were tested using generalized linear models (GLM) with binomial error distribution and logit link function. The effects of location of samples, season and size specimens were added into a full model and the minimal acceptable model was derived by backward elimination from a full model that included all terms

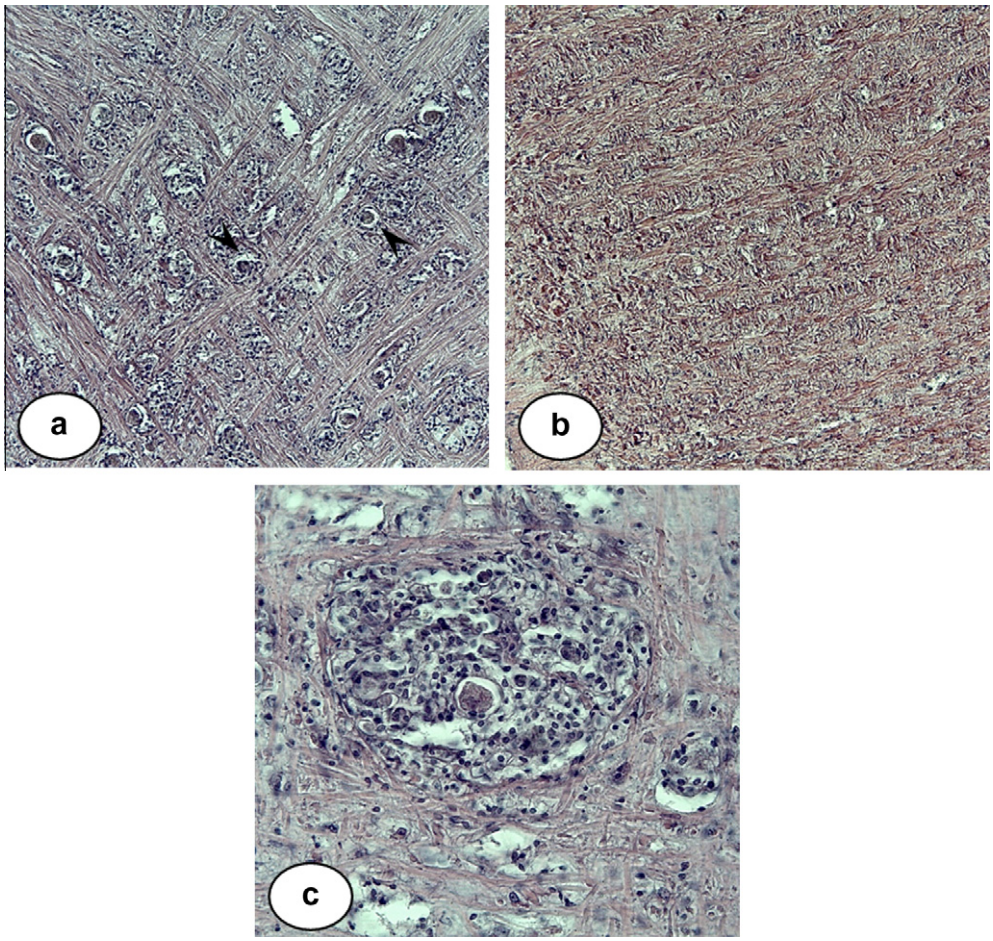

Fig. 3. Histological sections (light microscopy) of siphonal tissues of *Panopea abbreviata* infected by the green algae (a) compared with uninfected one (b), and a detail of the aggregation of hemocytes infected with algal cells (c).

and interactions (Newey et al., 2005). GLM analysis was also used to test the effect of parasite presence, location, season and size on condition index. In this case, a Gaussian error distribution along with an identity link function was used. Confidence intervals for the estimated treatment means were obtained by a nonparametric bootstrapping routine by using 2000 replicates (Efron and Tibshirani, 1993). All analyses were carried out using R language (R Development Core Team, 2008).

3. Results

3.1. Macroscopic observations

From a total of 450 geoducks, 68% showed a green area colored in the distal portion of inner surface of siphons (1–4 cm in length) (Fig. 2a and b). Fifty percent these geoducks registered a moderate intensity of infection. The only specimen of a razor clam showing the green color was recorded in the San Matías Gulf; and none of the oysters and scallops showed green coloration.

3.2. Histopathology of the green alga

Microscopically, histological sections of green colored siphonal tissues showed dense aggregations of approximately 65 μm diameter (SE = 15.2 μm, $n = 10$), distorting the orientation of the muscle and connective fibers (Fig. 3a and b), and containing colonies of algae, cellular debris, and infiltration by hemocytes, which were encapsulating the alga cells (Fig. 3c). Examination of histological sections of other tissues (gill, digestive gland, mantle, nephridia and gonad) failed to detect algal cells.

3.3. Algal morphology

At TEM, algae showed a round to oval shape, measuring 3.23 μm (SE = 0.11) in length and 2.44 μm (SE = 0.1) in width ($n = 8$). Up to six *Coccomyxa* cells ensheated in a common membrane were observed in a single hemocyte (Fig. 4a). In other cases, infected hemocytes included algal cells free within the cytoplasm, i.e., not surrounded by a membrane. Each algal cell contained one or two chloroplasts showing stacks of thylakoids and starch granules, mitochondria, and nucleus with one nucleolus (Fig. 4b). Two daughter cells or autospores inside the parental membrane were frequently observed (Fig. 4c). Occasionally, algal cells disintegrating inside a digestive vacuole in the cytoplasm of hemocytes were observed; in these cases two membranes, the algal cell membrane and the membrane of the digestive vacuole, were seen (Fig. 4d); but most algal colonies appeared highly resistant to digestion.

3.4. Phylogenetic position

Phylogenetic analysis based on a partial SSU rRNA sequencing was constructed including the information from green algae infecting *P. abbreviate*, and other marine and freshwater trebouxiophyceans, green endosymbionts in lichens and land plants (Fig. 5). Green algae infecting *P. abbreviata* from Argentina were clustered together with *C. parasitica* sequences (2–4 different nucleotides (nt)) obtained from mussels (*M. edulis chilensis*) from the North Sea. The separate branching pattern of the *C. parasitica* group from a sister clade including other *Coccomyxa* species and related endophytes was supported by moderate (>70) bootstrap values with the exception of the MP method.

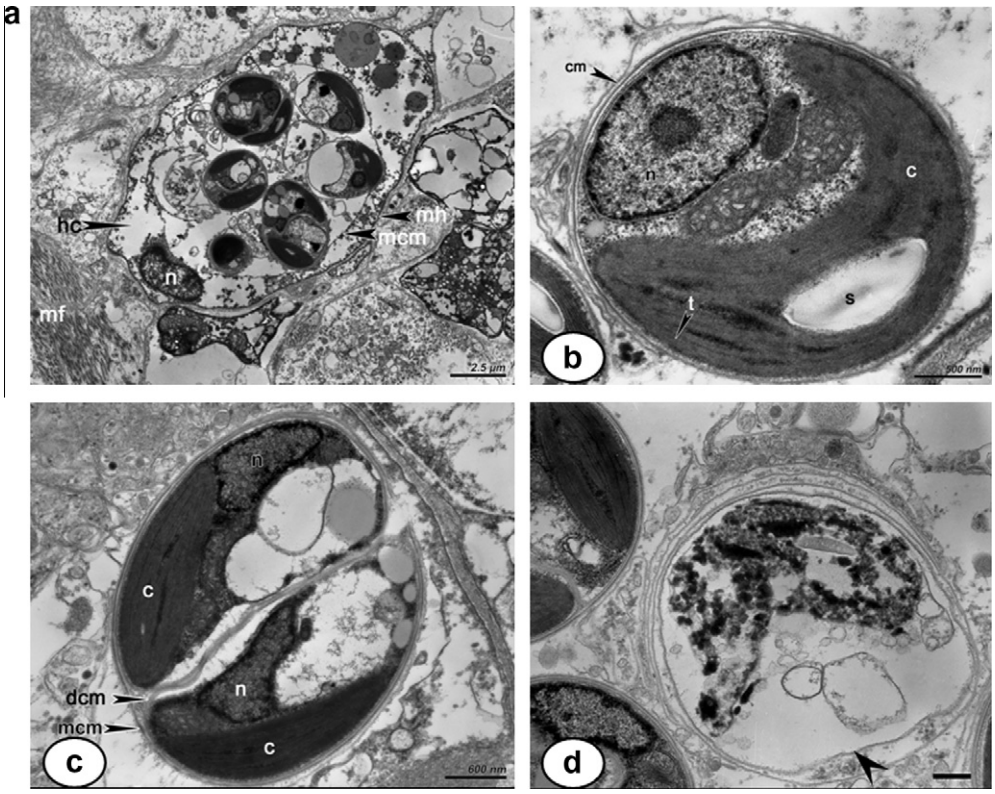

Fig. 4. TEM photographs of algal cells inside hemocytes of *Panopea abbreviata*. a. Six algal cells sheathed in: (a) common membrane within the hemocyte cytoplasm; (b) detail of one algal cell; (c) two daughter cells inside the parental membrane, and (d) disintegrating algal cell inside a digestion vacuole (arrow) within the hemocyte cytoplasm. References: c: chloroplast; cm: cellular membrane; dcm: algal daughter cell membrane; hc: hemocyte cytoplasm; mc: membrane of hemocyte; mcm: mother cell membrane; mf: muscular fiber; n: nucleus; s: starch; t: thylakoids.

3.5. Relation between algal prevalence, seasonal and location variations, and effects on the condition index of geoducks

For prevalence analysis, the results indicated significant effects of main factors location ($p < 0.001$) and season ($p < 0.001$) and their interactions ($p < 0.01$). The mean prevalence for geoducks from San Matías Gulf was 82% and those from San José Gulf was 54%. Along the year, specimens from San Matías Gulf showed prevalence values higher than those from San Jose Gulf, except for the spring season, where the two locations showed no differences (Fig. 6). Among seasons, no differences were found in geoducks from San Matías Gulf, but those from San José Gulf showed higher prevalences in spring and the lower values in autumn (Fig. 6). The interaction between size and location evidenced that in San Matías Gulf larger geoducks are more likely to be parasitized than smaller ones within the range of shell length of the geoducks sampled (59–116 mm) while the opposite effect was observed for those from San José Gulf (62–142 mm). For condition index analysis, the results indicated significant effects of location ($p < 0.0001$), season ($p < 0.01$), size ($p < 0.05$) and algal infection ($p < 0.0001$). In addition, only the

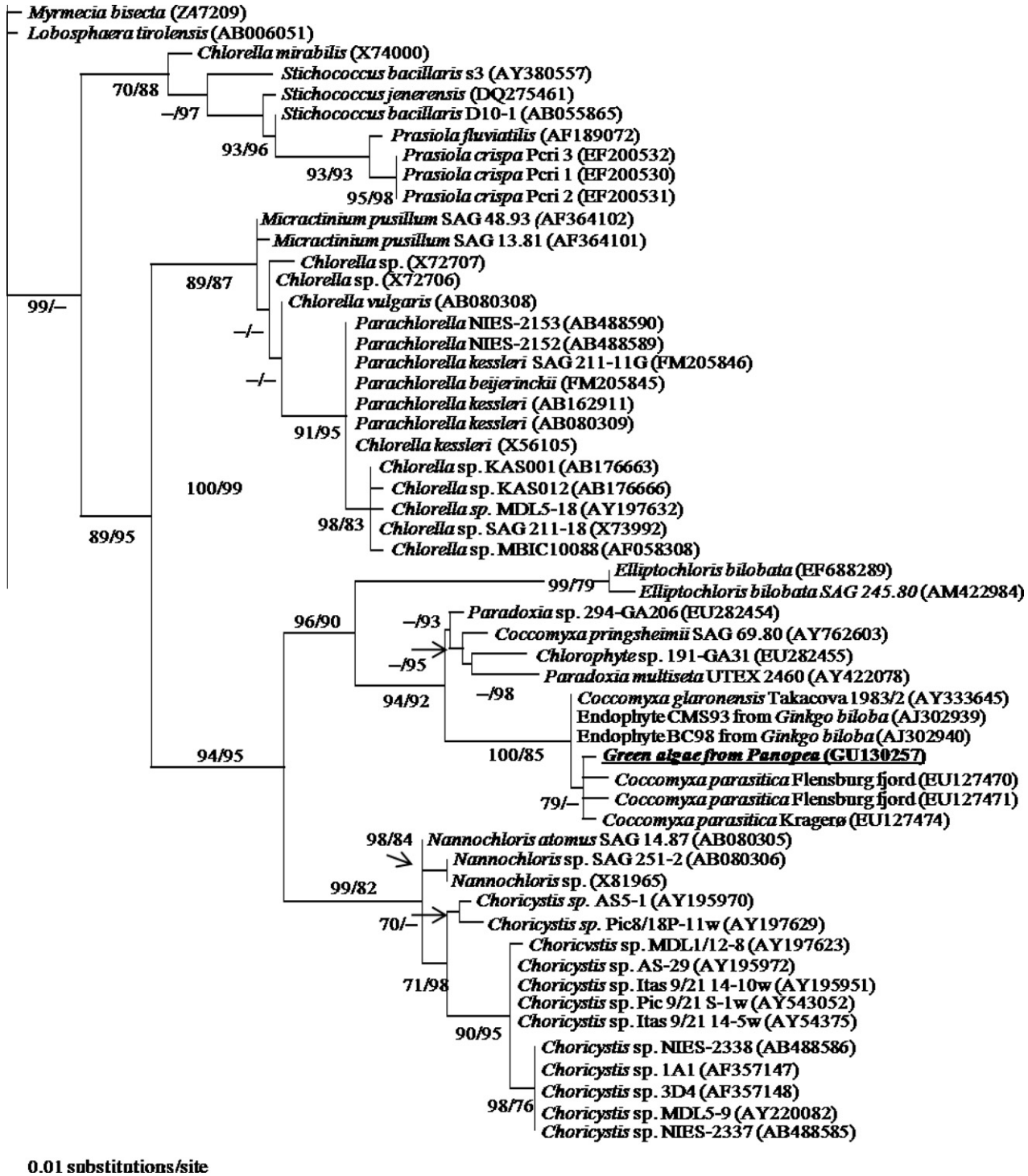

Fig. 5. Phylogenetic position of *Coccomyxa parasitica* found in Patagonian clams.

interaction between season and location was detected as significant ($p < 0.0001$). It is worth noting that the absence of interactions including the *C. parasitica* presence factor, implies that independently of location, season and size, the infected geoducks showed a lower condition index than the uninfected ones (Fig. 7).

4. Discussion

SSU rRNA phylogeny including sequences from green algae in *M. edulis* previously studied by Rodríguez et al. (2008) obtained in the present study, showed that the algae infecting *P. abbreviata* belongs to a *Coccomyxa*-like clade. Rodríguez et al. (2008) concluded, after comparison of morphological and histopathological results, that the green algae from *M. edulis* in the North Sea and Malvinas (Falkland) Islands would correspond with the original description of *C. parasitica* by Naidu and South (1970). As there were no available GenBank records from *C. parasitica,* sequences obtained by Rodríguez et al. (2008) were the only sequences for this species known to date. We did not include in our phylogeny the sequences from Malvinas (Falkland) Islands because they only shared 310 nt with our alignment. The resulting SSU rRNA phylogeny for such alignment would not be satisfactory, as it could not separate the *C. parasitica* sequences from other related taxa, not even *Paradoxia bilobata* (data not shown).

Even though *C. parasitica* has been previously reported in several bivalves as mussels and scallops, in our study none of the oysters and scallops examined was infected by this alga. This study is the first report recording *C. parasitica* in the geoduck *P. abbreviata,* and in the razor clam *E. macha*. Nevertheless, the razor clam would be acting as an accidental host, since the alga was found in only one out of 480 specimens examined. This seems related to the degree of the exposure of tissues to light, since it is vital for algal survival. Even though *P. abbreviata* and *E. macha* are both infaunal clams, the siphons of the latter are not exposed above the sediment surface. Thus, the photosynthetic metabolism of the algae inside its tissues would be limited due to the very low, if any, available irradiance. On the contrary, *P. abbreviata* extends their siphons about 20 cm above the sediment–water interface, resulting in an adequate exposure to light for algal survival. Light has been considered to be an important factor controlling the distribution and abundance of *C. parasitica* within the tissues of the scallop *P. magellanicus* (Naidu, 1971). Although *C. parasitica* was found distributed throughout the host tissues in *M. edulis chilensis* and *P. magellanicus*

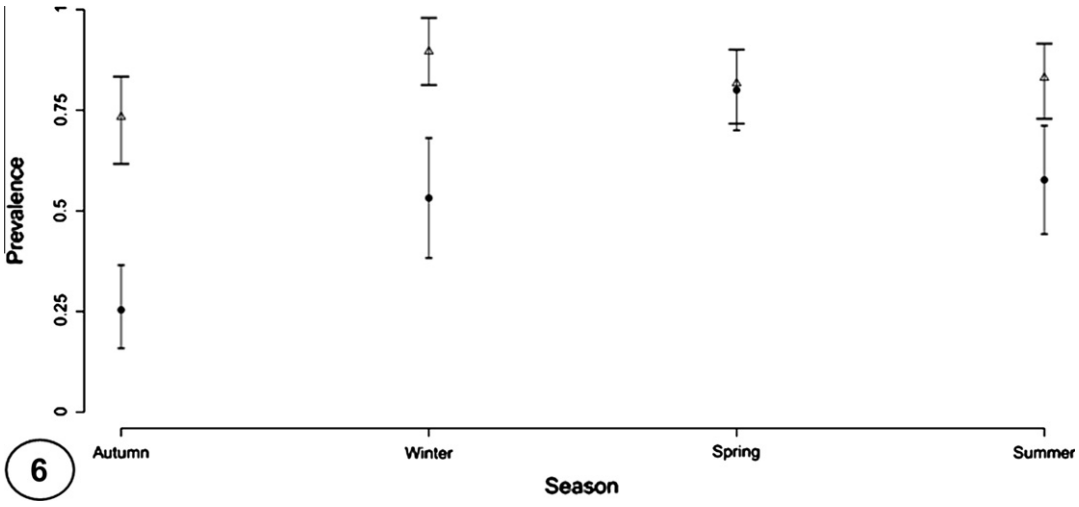

Fig. 6. Locations mean prevalence values across seasons for *Coccomyxa parasitica* in *Panopea abbreviata*. References: triangles and circles points correspond to San Matías Gulf and San José Gulf prevalence means respectively. Vertical bars indicate the 95% bootstrap confidence intervals by using 2000 replicates.

Fig. 7. Effect of the presence of *Coccomyxa parasitica* on the condition index in *Panopea abbreviata*. Vertical bars indicate the 95% bootstrap confidence intervals by using 2000 replicates.

(Naidu, 1971; Gray et al., 1999), in *P. abbreviata* the alga establishes and reproduces only inside the hemocytes and occasionally in cells of the connective tissue of the terminal portion of siphons.

Both higher nutrient concentrations and higher density of natural beds of geoducks in Puerto Lobos (San Matías Gulf) (Ciocco et al., 2004; Gagliardini and Rivas, 2004), could be determining the higher prevalences of *C. parasitica* in *P. abbreviata* found in this location compared with the lower values in Punta Conos (San José Gulf). The area of Puerto Lobos receives water with high concentrations of phytoplankton during the whole year (Gagliardini and Rivas, 2004), that might been supplying *Coccomyxa* algal cells; whilst the area of Punta Conos shows a peak of phytoplankton concentration only during the spring season.

Even though the type of association between *C. parasitica* and their bivalve host is not fully clear, the algal infection causes pathological alterations in the host tissues. The presence of the green algae caused damage to the geoduck tissues by eliciting disruptions and disorders in the arrangement of muscle and connective fibers of the siphons. There was also hemocytic infiltration in infected tissues and phagocytic processes, which may act as a drain on energy. Cheng (1976) pointed out that the energy for phagocytosis in the clam *Mercenaria mercenaria*, appeared to be derived from a glycolytic pathway, since the hemocytes utilize glucose and glycogen. The results obtained in the present work support this hypothesis by the fact that the lowest condition index was statistically associated to the infected geoducks. This suggests that *C. parasitica* might have an adverse effect on health of *P. abbreviata* which signifies the parasitic nature of this alga.

Acknowledgments

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