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Molecular evidence for *Chondrophycus poiteaui* var. *gemmiferus* comb. *et* stat. nov. (Ceramiales, Rhodophyta) from the Mexican Caribbean Sea: implications for the taxonomy of the *Laurencia* complex

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Abstract

Molecular studies were carried out on *Chondrophycus gemmiferus* and *C. poiteaui* (Rhodomelaceae) from the Mexican Caribbean Sea. These species are morphologically related, but differ mainly in the presence of the apiculate projection of epidermal cells near the apices of branches. Both species belong to *Chondrophycus,* as indicated by the presence of two periaxial cells per axial segment and a 90° arrangement of tetrasporangia, but share characteristics with *Laurencia* species (e.g., presence of secondary pit connections between adjacent epidermal cells). The phylogenetic position of these species was inferred by an analysis of chloroplast-encoded *rbc*L gene sequences of 21 taxa, using two members of the Rhodomelaceae and two of the Ceramiaceae as outgroups. The results corroborate the taxonomy of the *Laurencia* complex, which comprises the genera *Laurencia, Chondrophycus* and *Osmundea*, and indicate that *rbc*L provides an adequate phylogenetic signal to study the intergeneric and interspecific relationships within the complex. In spite of this, relationships within the clade formed by *C. gemmiferus* and *C. poiteaui* were not resolved by any analysis because of the low level of genetic variation between their *rbc*L sequences (0.01–0.02%). On the basis of both molecular data and morphological similarities, we concluded that *C. gemmiferus* should be considered as a variety of *C. poiteaui* and the following new combination is proposed: *Chondrophycus poiteaui* var. *gemmiferus* (Harvey) comb. *et* stat. nov.

Keywords: Ceramiales; *Chondrophycus poiteaui*; Mexican Caribbean Sea; phylogeny; *rbcL*.

Introduction

Significant changes in the classification of *Laurencia* J.V. Lamouroux (1813) have taken place during the last two decades. At present, there is a consensus that the *Laurencia* complex is formed by three genera: *Laurencia sensu stricto, Chondrophycus* (Tokida *et* Saito) Garbary *et* J. Harper (1998) and *Osmundea* Stackhouse (Nam et al. 1994). The basic differences among these genera are the number of periaxial cells per vegetative axial segment, the origin and arrangement of the tetrasporangia, the origin and type of the spermatangial branches and presporangial cells on the fertile branches (Nam et al. 1994, Garbary and Harper 1998, Nam 1999, Furnari et al. 2001).

The present taxonomic status of *Chondrophycus gemmiferus* (Harvey) Garbary *et* J. Harper and *C. poiteaui* (J.V. Lamour.) K.W. Nam was established by Nam (1999), who placed these species within *Chondrophycus*, based on the presence of two periaxial cells per vegetative axial segment, a trichoblast-type spermatangial branch, spermatangial branches produced from one of two laterals on suprabasal cell of trichoblasts, procarp-bearing segments with four or five pericentral cells, tetrasporangial production from particular pericentral cells, tetrasporangial axis with one sterile pericentral cell, the second pericentral cell being fertile. The two species were placed in the subgenus *Yuzurua* K.W. Nam, because of the presence of secondary pit connections between epidermal cells, one sterile periaxial cell in tetrasporangial axial segments and a 90° arrangement of the tetrasporangia. Finally, *C. gemmiferus* was included in the section *Parvipapillatae* K.W. Nam, because of the presence of projections from epidermal cells at the apices of the branchlets, while *C. poiteaui* was included in the section *Yuzurua*, as projections from epidermal cells at the apices of the branchlets are absent.

Chondrophycus gemmiferus and *C. poiteaui* are typical members of tropical western Atlantic Ocean flora, and are common and abundant in the Mexican Caribbean Sea (Sentíes and Fujii 2002, Wynne 2005). Fujii et al. (1996) found them growing sympatrically in the Mexican Nichupté Lagoon system, Quintana Roo State. Both species are morphologically similar and characterized by the presence of a clearly discernible main axis with small ultimate branchlets. The species were originally described from Key West, FL, USA and Santo Domingo, Dominican Republic, Greater Antilles, respectively.

Yamada (1931) made an important contribution to the understanding of the genus *Laurencia* through detailed examinations of several type materials. In his account,

he treated *Laurencia gemmifera* Harvey as a variety of *Laurencia poiteaui* (J.V. Lamouroux) Howe ("*poitei*"), proposing the combination, *L. poiteaui* ("*poitei*") var. *gemmifera* (Harvey) Yamada. Fujii et al. (1996) recognized that *L. gemmifera* and *L. poiteaui* have strong similarities in overall appearance, sharing several characteristics, such as presence of secondary pit connections between adjacent cortical cells and 90° arrangement of tetrasporangia. Nevertheless, *L. gemmifera* was differentiated from *L. poiteaui* by the presence of apiculate cortical cells near the apices of branches, and in having smaller ultimate branchlets, so that the species were maintained as independent entities, ignoring Yamada's (1931) taxonomic treatment.

Wynne (1998, 2005) accepted Fujii and collaborators' argument (Fuji et al. 2006) in his checklists of benthic marine algae of the tropical and subtropical western Atlantic Ocean. In contrast, Silva et al. (1996) recognized the trinomial epithet, *Laurencia poiteaui* var. *gemmifera*. Nam (1999) transferred *L. gemmifera* and *L. poiteaui* from *Laurencia* to *Chondrophycus* on the basis of the presence of two pericentral cells per vegetative axial segment.

Important proposals on the classification system of the *Laurencia* complex have been made on the basis of phylogeny, taking into account mainly morphological attributes (Garbary and Harper 1998, Nam 1999, 2006). Currently, only four papers have been based on molecular phylogeny (Nam et al. 2000, McIvor et al. 2002, Abe et al. 2006, Fujii et al. 2006). The study by Nam et al. (2000) assessed the phylogenetic significance of several morphological characters in *Osmundea* by comparative morphological and molecular analyses, but was confined to the European species. The authors found that the European species of *Osmundea* separated into two groups, based on the presence or absence of secondary pit connections.

Fujii et al. (2006) studied the phylogenetic affinities of the red alga, *Chondrophycus flagelliferus*, from Brazil using morphological and molecular evidence. The molecular data indicated that *C. flagelliferus* is closely related to the *C. papillosus* complex, and that, as originally described, *C. translucidus* belongs to the genus *Laurencia*. The relationships among the *L. scoparia*, *L. arbuscula* and *L. filiformis* have not been resolved because of the absence or lower levels of genetic variation observed among the sequences of these species. All species currently placed in the genus *Osmundea* form a monophyletic clade. However, two clades representing different geographical areas were observed (North Pacific and Atlantic Europe), corroborating the distinct biogeographic pattern within the genus, as observed by McIvor et al. (2002). Abe et al. (2006) carried out a molecular phylogenetic analysis of three closely related red algal genera, *Laurencia*, *Chondrophycus* and *Osmundea* (mainly on the north-western Pacific species). The results showed that *Osmundea* and *Laurencia sensu stricto* were monophyletic and *Chondrophycus* was polyphyletic within the *Laurencia* complex. *L. flexilis* (an intermediate species between typical *Laurencia* and *Chondrophycus*) constituted an independent monophyletic clade with high bootstrap values. Nam's (2006) cladistic analysis using morphological characters also demonstrated that *Chondrophycus*, as currently defined, is not monophyletic. Consequently, he proposed recognition of the segregate genus *Palisada*, but that generic name was not validated (P. Silva and M. Wynne, personal communication).

This is the first paper of its type using Mexican material; we assessed the taxonomic status of *Chondrophycus gemmiferus* and *C. poiteaui* by analyzing the nucleotide sequences of the *rbc*L gene of both species. That led us to formulate taxonomic proposals and to determine the phylogenetic positions of these two species within the *Laurencia* complex. Fujii et al. (1996), Nam et al. (2000) and McIvor et al. (2002) indicated that the *rbc*L gene provides sufficient phylogenetic signal to determine the intergeneric and interspecific relationships within the complex.

Materials and methods

Sample collection

Two individuals of each species were collected from several localities in the Mexican Caribbean Sea (from 18°11′ to 21°54′ N and from 86°15′ to 87°54′ W), as reported previously by Sentíes and Fujii (2002), and dried in silica gel. The specimens used for molecular analysis are shown in Table 1, including their GenBank accession numbers (NCBI GenBank 2003).

Extraction and amplification of DNA

Total DNA was extracted, after grinding in liquid nitrogen, using the Dneasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. A total of 1467 base pairs of the *rbc*L gene was amplified in three parts with the primer pairs: FrbcLstart×R753, F577×R1150 and F753×RrbcS (Freshwater and Rueness 1994) using the master mix of the Bioneer (Daedeok-Gu, Daejeon, Korea) Premix. The conditions for amplification were 4 min at 96°C for a hot start, 35 cycles of 60 s at 94°C, 60 s at 42°C and 90 s at 72°C, with a final extension of 10 min at 72°C. The reactions were kept at 4°C after amplification. All PCR products were analyzed by electrophoresis in 1% agarose to check product size. The PCR products were purified with the Qiagen QIAquick purification kit (Qiagen) in accordance with the manufacturer's instructions.

Sequencing

Sequencing was performed using the BigDye terminator cycle sequencing reaction kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Princeton, NJ, USA). The primers used for sequencing were those used for amplification. The full sequence was obtained from both DNA strands. Analysis of the sequences was performed using the computer program Sequencer Navigator (Applied Biosystems), and was aligned with the CLUSTAL algorithm (Thompson et al. 1994).

Table 1 Species used in this study for phylogenetic analysis.

Sample	Location	Collectors	GenBank accession no.
Bryocladia cuspidata (J. Agardh) De Toni[b]	Port Aransas, TX, USA	S. Fredericq and C.F.D. Gurgel	AF259498
Centroceras clavulatum (C. Agardh) Montagne[b]	USA	S. Fredericq and C.F.D. Gurgel	AF259490
Ceramium brevizonatum Petersen[b]	USA	–	AF259491
Chondria dasyphylla (Woodward) C. Agardh[b]	Morehead City, NC, USA	D.W. Freshwater	U04021
Chondrophycus corallopsis (Montagne) Nam[a]	Chaac-Mol Beach, Cancun, Quintana Roo, Mexico	J. Díaz and A. Sentíes	EF061646
C. flagelliferus (J. Agardh) Nam[a]	Es. Marataizes, Espírito Santo, Brazil	M.T. Fujii	EF061647
C. flagelliferus (J. Agardh) Nam	Ubatuba, Praia Brava, Sao Paulo, Brazil	S.M.P.B. Guimarães and J. Domingos	AF465804
C. gemmiferus (Harvey) Garbary *et* Harper[a]	Ojo Agua, Puerto Morelos, Quintana Roo, Mexico	J. Díaz and A. Sentíes	EF061648
C. gemmiferus (Harvey) Garbary *et* Harper[a]	Playa del Carmen, Cancun, Quintana Roo, Mexico	J. Díaz and A. Sentíes	EF061649
C. gemmiferus (Harvey) Garbary *et* Harper[a]	Rincon de Guanabo, La Havana, Cuba	J. Díaz and A. Areces	EF061650
C. papillosus (C. Agardh) Garbary *et* Harper[a]	CRIP, Puerto Morelos, Quintana Roo, Mexico	J. Díaz and A. Sentíes	EF061651
C. papillosus (C. Agardh) Garbary *et* Harper	Old Dan's Reef, Keys Marine Lab., FL, USA	C.F.D. Gurgel	AF465807
C. poiteaui (Lamouroux) Nam[a]	Ocean Side, Long Key, FL, USA	S. Fredericq	EF061652
C. poiteaui (Lamouroux) Nam[a]	Playa del Carmen, Quintana Roo, Mexico	J. Díaz and A. Sentíes	EF061653
Laurencia brongniartii J. Agardh	Makang Harbor, Taiwan	S. Fredericq	AF465814
L. obtusa (Hudson) Lamouroux	Pointe de la Verdure, Guadeloupe	A. Renoux	AF465811
L. obtusa (Hudson) Lamouroux	Fanad Head, County Donegal, Ireland	–	AF281881
L. venusta Yamada[a]	Punta Brava, Puerto Morelos, Quintana Roo, Mexico	J. Diaz and A. Sentíes	EF061655
Osmundea osmunda (S.G. Gmelin) Nam *et* Maggs	Ireland	–	AF281877
O. pinnatifida (Hudson) Stackhouse	St. John's Point, County Donegal, Ireland	–	AF281875
O. truncata (Kützing) Nam *et* Maggs	Ireland	–	AF281879

[a]Sequences obtained in this work. [b]Outgroups.

Phylogenetic analysis

Phylogenetic relationships were inferred with PAUP* 4.0b10 (Swofford 2002). Maximum parsimony (MP) trees were constructed using the heuristic search option, tree-bisection-reconnection branch swapping, unordered and unweighted characters, and gaps treated as missing data. The ModelTest version 3.7 program (Posada and Crandall 1998) was used to find the model of sequence evolution least rejected for each data set by a hierarchical likelihood ratio test. When the evolution model had been determined, maximum likelihood searches were performed using the estimated parameters (substitution model, gamma distribution, proportion of invariant sites, frequencies of the bases). Maximum likelihood (ML) was used to construct the most likely tree from the data set.

Support for individual internal branches was determined by bootstrap analysis (Felsenstein 1985), as implemented in PAUP*. For bootstrap analysis, 1000 bootstrap data sets were generated from re-sampled data for the MP analysis and 100 replicates for the ML analysis.

The consistency (CI), homoplasy (HI) and retention (RI) indices resulting from the MP analysis were calculated. Outgroup species were selected on the basis of close phylogenetic relationship with the ingroup. The range of *rbc*L divergence values within and among species was calculated using uncorrected "p" distances using PAUP*.

Results

Variation in the *rbc*L sequences

A total of 21 sequences was analyzed including the outgroups *Ceramium brevizonatum*, *Centroceras clavulatum*, *Chondria dasyphylla* and *Bryocladia cuspidata* (Table 1). We fully sequenced the *rbc*L gene from 9 taxa of the *Laurencia* complex. The first 60 nucleotides of all *rbc*L sequences were removed, producing a data set of 1407 base pairs, and the rest of the sequences were aligned without ambiguity.

Intergeneric divergence varied from 10 to 12% for *Chondrophycus* and *Laurencia* and from 10 to 13% for *Chondrophycus* and *Osmundea*. Interspecific divergence obtained for the species of *Laurencia* varied from 6 to 8%, whereas for those of *Chondrophycus* it varied from 6 to 9%. In the case of *C. gemmiferus* and *C. poiteaui*, the observed nucleotide divergence within *rbc*L gene sequence was very low (0.01–0.02%).

Phylogeny

The data set consisted of 927 constant characters, 179 parsimony informative sites and 344 parsimony non-informative sites. MP produced 4 trees of 1663 steps (CI=0.574, RI=0.597, HI=0.426). The topology of strict consensus of these trees with corresponding bootstrap values is shown in Figure 1.

Maximum likelihood (estimated evolution model: substitution model=GTR+I+G; gamma distribution=1.1024; proportion of invariant sites=0.4739; frequency of the bases was: A=0.3270, C=0.1439, G=0.2009 and T=0.3282; the rate matrix was: [A–C]=4.7158, [A–G]=6.1517, [A–T]=4.8734, [C–G]=1.075, [C–T]=33.1711) produced a topology of -ln L score of 3243.1849. The topology of maximum likelihood phylogram derived from *rbc*L sequences with corresponding bootstrap values is shown in Figure 2.

MP and ML topologies were not significantly different. The analyses show a monophyletic *Laurencia* complex with high bootstrap support in relation to the members of the outgroups. The *Laurencia* complex separated into three clades with high bootstrap support, corresponding to the genera that form the complex: *Laurencia, Chondrophycus* and *Osmundea.* In all the analyses, the earliest diverging clade was the genus *Osmundea* (with its species *O. pinnatifida*, *O. osmunda* and *O. truncata*), and

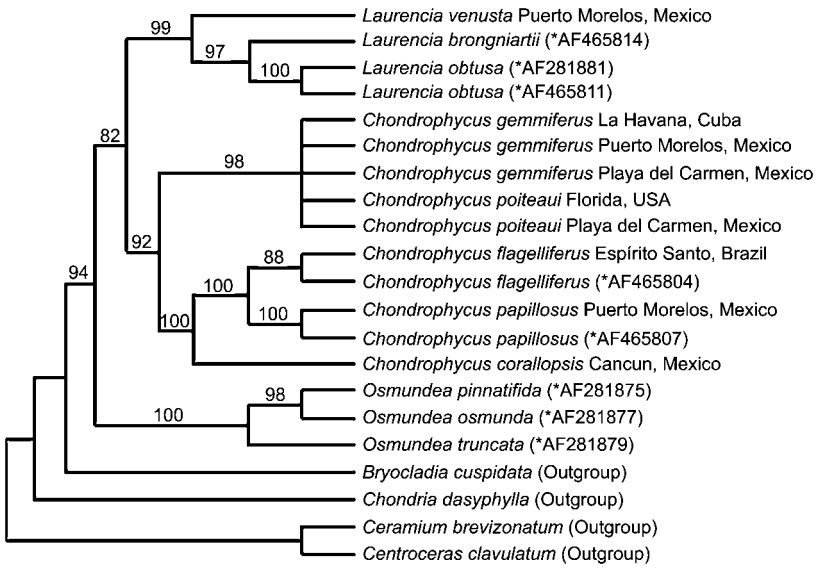

Figure 1 Strict consensus of four maximum parsimony trees for *rbc*L sequences of the *Laurencia* complex species rooted with *Centroceras clavulatum*, *Ceramium brevizonatum*, *Chondria dasyphylla* and *Bryocladia cuspidata.*
Bootstrap values (1000 replicates) are indicated at the nodes. (*) GenBank sequences.

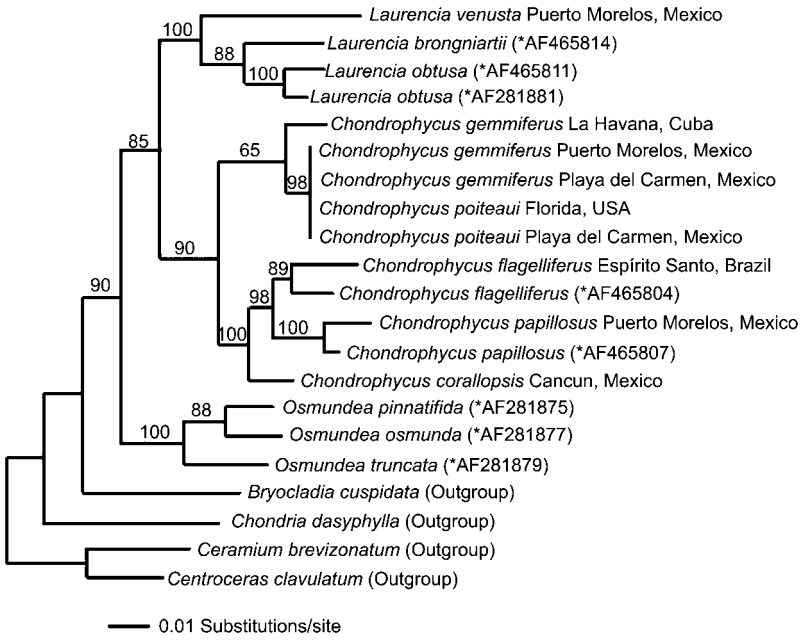

Figure 2 Maximum likelihood phylogram derived from *rbc*L sequences of the *Laurencia* complex species rooted with *Centroceras clavulatum, Ceramium brevizonatum*, *Chondria dasyphylla* and *Bryocladia cuspidata.*
Bootstrap values (100 replicates) are indicated at the nodes. (*) GenBank sequences.

the genera *Laurencia* and *Chondrophycus* were sister groups forming a clade with high bootstrap support.

The monophyletic clade that corresponded to the genus *Chondrophycus* included five species. Within the *Chondrophycus* assemblage, *C. gemmiferus* and *C. poiteaui*, were segregated from others forming a distinct clade. The species *C. flagelliferus*, *C. papillosus* and *C. corallopsis* formed another well-supported clade.

The monophyletic clade for the genus *Laurencia* included three species. *L. venusta* was a sister species to the other two and is the only species with verticillate ramification. *L. brongniartii* and *L. obtusa* formed one group in which *L. brongniartii* is the only species with a compressed thallus.

Discussion

Chondrophycus gemmiferus and *C. poiteaui* share some characteristics, i.e., the presence of secondary pit connections between epidermal cells, one sterile periaxial cell in tetrasporangial axial segments, and a 90° arrangement of the tetrasporangia. The only difference between them is the presence of projections from epidermal cells at the apices of the branchlets in *C. gemmiferus* and the absence of these in *C. poiteaui* (Fujii et al. 1996, Sentíes and Fujii 2002).

This last characteristic, together with a detailed morphological description of populations of these species from the Mexican Caribbean Sea by Fujii et al. (1996), made it possible to conclude that *Chondrophycus gemmiferus* and *C. poiteaui* should remain as independent entities, notwithstanding the fact that there are more morphological similarities than differences. *C. gemmiferus* has smaller ultimate branchlets than *C. poiteaui* (Sentíes and Fujii 2002). However, between the species there are minimum variations in ramification throughout the thallus, which does not support segregation at the species level. Also, anatomical and reproductive characteristics differ little with respect to the meristic nature of medullary and epidermal cells, and in male, female and tetrasporangial structures (Sentíes and Fujii 2002). Fujii et al. (1996) also emphasized that these species show a mix of characters between the genera *Chondrophycus* and *Laurencia*, such as the presence of two periaxial cells per vegetative axial segment (corresponding to *Chondrophycus*), and the presence of secondary pit connections between epidermal cells (more characteristic of *Laurencia*).

Other species that share characteristics with *Chondrophycus gemmiferus* and are placed in the section Parvipapillatae are *C. parvipapillatus* (C.K. Tseng) Garbary *et* J.T. Harper from China and *C. iridescens* (M.J. Wynne *et* D.L. Ballantine) K.W. Nam from Guadeloupe and Puerto Rico in the Caribbean Sea. Differences between them are the presence of a diminutive compressed thallus in *C. parvipapillatus* (2 cm), an iridescent thallus in *C. iridescens*, and a cylindrical thallus, reaching $\leq$15 cm and no iridescence in *C. gemmiferus* (Wynne and Ballantine 1991, Nam 1999).

The intergeneric divergence values obtained are comparable to those reported by other authors for the *Laurencia* complex. Nam et al. (2000) recorded intergeneric divergence values between 11% (*L. obtusa* vs. *Osmundea pinnatifida*) and 13% [*L. obtusa* vs. *O. hybrida* (A.P. de Candolle) W.K. Nam], and McIvor et al. (2002) recorded values of 11% (*O. pinnatifida* vs. *L. obtusa*) to 13% [*O. blinksii* (Hollenberg *et* I.A. Abbott] K.W. Nam vs. *Chondrophycus papillosus*).

The interspecific divergence values are comparable to those reported by other authors for the genus *Osmundea*. Nam et al. (2000) estimated divergence percentages that varied from 5% (*O. osmunda* vs. *O. pinnatifida*) to 9% (*O. hybrida* vs. *O. truncata*), and McIvor et al. (2002) recorded values of 2% (*O. blinksii* vs. *O. splendens*) to 9% (*O. blinksii* vs. *O. truncata*).

The molecular phylogeny shows that the clade formed by the three populations (Puerto Morelos, Mexico; Playa del Carmen, Mexico; and La Havana, Cuba) of *Chondrophycus gemmiferus* and two of *C. poiteaui* (Playa del Carmen, Mexico and Florida, USA) are poorly resolved as a result of the low level of genetic variation between their sequences (0.01–0.02%). In contrast, the sister clade comprising the species *C. flagelliferus*, *C. papillosus* and *C. corallopsis* is well resolved based on molecular data from *rbc*L. This conforms to the proposal of Fujii et al. (2006), who suggest that *C. flagelliferus* is sister to the *C. papillosus* complex. These species are also easily distinguishable on morphological characteristics, such as the presence of a palisade-like arrangement of the epidermal cells in transverse section of the branch in *C. flagelliferus*, the presence of short papilliform branchlets on each branch in *C. papillosus*, and the presence of prominent cystocarps and a corymbose-like ramification in *C. corallopsis* (Sentíes and Fujii 2002).

Similar to Garbary and Harper (1998) and Fujii et al. (2006), our results obtained through the analysis of *rbc*L confirm the existence of the *Laurencia* complex as a monophyletic clade that includes the genera *Chondrophycus*, *Laurencia* and *Osmundea* separated into clearly defined monophyletic clades. *Laurencia* and *Chondrophycus* appear as sister groups, with the separation of the two genera confirmed in our analysis by the presence of four and two periaxial cells per axial segment, respectively.

Records from the type localities of *Chondrophycus gemmiferus* (Key West, FL, USA) and of *C. poiteaui* (Santo Domingo, Greater Antilles, Caribbean Sea) and other nearby areas (Wynne 2005) make it possible to establish that these species co-exist in tropical and subtropical regions of the western Atlantic Ocean, with the Mexican Caribbean Sea as the area with the greatest abundance of these sympatric species (Fujii et al. 1996, Sentíes and Fujii 2002). In consequence, their geographical distribution does not support a formal separation of the species, and local aspects of habitats and environments are identical or similar among the species.

In conclusion, considering the almost identical *rbc*L sequences of *Chondrophycus gemmiferus* and *C. poiteaui*, the very similar morphological characters and the sympatric distribution, we agree with the taxonomic treatment of Yamada (1931) of the above taxa proposing the following new combination:

- *Chondrophycus poiteaui* (J.V. Lamouroux) K.W. Nam var. *gemmiferus* (Harvey) Sentíes, Fujii *et* Díaz comb. *et* stat. nov.
- Basionym: *Laurencia gemmifera* (Harvey 1853, Nereis Boreali-Americana, vol. 2, p. 73, Tab. XVIII, B).
- Homotypic synonyms: *Laurencia poiteaui* (J.V. Lamouroux) M. Howe var. *gemmifera* (Harvey) Yamada. *Chondrophycus gemmiferus* (Harvey) Garbary *et* J. Harper.

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