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Molecular systematics of *Thorea* (Rhodophyta, Thoreales) species in Brazil

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ABSTRACT – (Molecular systematics of *Thorea* (Rhodophyta, Thoreales) species in Brazil). This study aimed to evaluate species level taxonomy and phylogenetic relationship among *Thorea* species in Brazil and other regions of the world using two molecular markers – RUBISCO large subunit plastid gene (*rbc*L) and nuclear small-subunit ribosomal DNA (SSU rDNA). Three samples of *Thorea* from Brazil (states of Mato Grosso do Sul and São Paulo) and one sample from Dominican Republic (DR) were sequenced. Analyses based on partial sequences of *rbc*L (1,282 bp) and complete sequences of SSU (1,752 bp) were essentially congruent and revealed that Thoreales formed a distinct monophyletic clade, which had two major branches with high support, representing the genera *Thorea* and *Nemalionopsis*. *Thorea* clade had four main branches with high support for all analyses, each one representing the species: 1) *T. gaudichaudii* C. Agardh from Asia (Japan and Philippines) – this clade occurred only in the *rbc*L analyses; 2) *T. violacea* Bory from Asia (Japan) and North America (U.S.A. and DR); 3) *T. hispida* (Thore) Desvaux from Europe (England) and Asia (Japan); 4) a distinct group with the three Brazilian samples (sequence identity: *rbc*L 97.2%, 1,246 bp; SSU 96.0-98.1%, 1,699-1,720 bp). The Brazilian samples clearly formed a monophyletic clade based on both molecular markers and was interpreted as a separate species, for which we resurrected the name *T. bachmannii* Pujals. Morphological and molecular evidences indicate that the Thoreales is well-resolved at ordinal and generic levels. In contrast, *Thorea* species recognized by molecular data require additional characters (*e.g.* reproductive and chromosome numbers) to allow consistent and reliable taxonomic circumscription aiming at a world revision based on molecular and morphological evidences.

Key words - freshwater Rhodophyta, *rbc*L, SSU rDNA, *Thorea*, Thoreales

RESUMO – (Sistemática molecular de espécies de *Thorea* (Rhodophyta, Thoreales) no Brasil). Este estudo objetivou avaliar a taxonomia no nível específico e as relações filogenéticas entre as espécies de *Thorea* do Brasil e de outras regiões do mundo usando dois marcadores moleculares – genes plastidial da subunidade grande da RUBISCO (*rbc*L) e nuclear da subunidade pequena do DNA ribossômico (SSU rDNA). Três amostras de *Thorea* do Brasil (Estados de Mato Grosso do Sul e São Paulo) e uma amostra da República Dominicana (RD) foram sequenciadas. Análises baseadas nas sequências parciais de *rbc*L (1.282 pb) e completas de SSU (1.752 pb) foram essencialmente congruentes e revelaram que Thoreales formou um clado monofilético distinto, que teve dois ramos principais com alto suporte, representando os gêneros *Thorea* e *Nemalionopsis*. O clado de *Thorea* teve quarto ramos principais com alto suporte em todas análises, cada um representando as espécies: 1) *T. gaudichaudii* C. Agardh da Ásia (Japão e Filipinas); este clado ocorreu apenas nas análises de *rbc*L; 2) *T. violacea* Bory da Ásia (Japão) e América do Norte (E.U.A. e RD); 3) *T. hispida* (Thore) Desvaux da Europa (Inglaterra) e Ásia (Japão); 4) um grupo distinto com as três amostras do Brasil (identidade das sequências: *rbc*L 97,2%, 1.246 pb; SSU 96,0-98,1%, 1.699-1.720 pb). As amostras brasileiras formaram claramente um clado monofilético baseado nos dois marcadores moleculares e foi interpretado como uma espécie distinta, para a qual restabelecemos o nome *T. bachmannii* Pujals. Evidências morfológicas e moleculares indicam que Thoreales é bem resolvida nos níveis ordem e gênero. Em contrapartida, as espécies de *Thorea* reconhecidas por dados moleculares requerem caracteres adicionais (p.ex. reprodutivos e números de cromossomos) para permitir circunscrição taxonômica consistente e confiável visando revisão mundial baseada em evidências moleculares e morfológicas.

Palavras-chave - gene *rbc*L, Rhodophyta continental, SSU rDNA, *Thorea*, Thoreales

Introduction

The genus *Thorea* was proposed by Bory de Saint-Vicent (1808) and has been classified in the family Thoreaceae of the order Batrachospermales (Sheath *et al*. 1993, Necchi Júnior & Zucchi 1997, Entwisle & Foard 1999). Thoreaceae is distinguished from the other members of the Batrachospermales basically by having multiaxial thalli (Sheath *et al*. 1993, Kumano 2002). The sequence data from the large subunit of ribulose 1,5-bisphosphate carboxilase/oxygenase (*rbc*L) and ribosomal DNA (small subunit, SSU rDNA) genes showed that *Thorea* does not appear to be a natural grouping within the Batrachospermales (Vis *et al*. 1998). The authors suggested that Thoreaceae should be elevated

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to ordinal status but still considered it as "incertae sedis" in the Florideophyceae. Müller *et al*. (2002) proposed the order Thoreales to accommodate the two recognized genera (*Thorea* and *Nemalionopsis*) based on DNA sequences of these two genes and ultrastructural features (pit plugs with two cap layers, the outer one typically plate-like). *Thorea* is distinguished by having assimilatory filaments not contained in a common gelatinous matrix with reproductive structures (carpogonia, spermatangia, carposporangia and monosporangia) at their base, whereas in *Nemalionopsis* they are embedded in a gelatinous matrix with reproductive structures at their apex (Sheath *et al.* 1993, Müller *et al.* 2002).

Members of the family are distributed worldwide but tend to be more common in tropical and subtropical regions or warm temperate waters (Sheath & Hambrook 1990, Sheath *et al*. 1993, Carmona & Necchi Júnior 2001). *Thorea* has been reported from several continents, whereas *Nemalionopsis* is known only from few localities in Asia and North America (Sheath *et al*. 1993, Müller *et al.* 2002). Only four species of *Thorea* were recognized worldwide by Sheath *et al*. (1993): *T. clavata* Seto *et* Ratnasabapathy, *T. hispida* (Thore) Desvaux, *T. violacea* Bory and *T. zollingeri* Schmitz. Entwisle & Foard (1999) described *T. conturba* from Australia. Kumano (2002) recognized six additional species species in the world: *T. bachmannii* Pujals, *T. brodensis* Klas, *T. gaudichaudii* C. Agardh, *T. okadae*Yamada, *T.prowsei* Ratnasabapathy *et* Seto and. *T. riekei* Bischoff. Taxonomic characters used for species delineation include essentially vegetative features: plant length, branching (abundant or sparse secondary branches), size and shape of assimilatory filaments (clavate or non-clavate), size of assimilatory filaments and frequency of monosporangial clusters. This fact usually brings problems for species circumscriptions in the genus, which are still not satisfactorily resolved. Sexual reproduction, as well as the presence of juvenile gametophytes, have also been observed under natural conditions (Yoshizaki 1986, Necchi Júnior 1987, Sheath *et al.* 1993, Necchi Júnior & Zucchi 1997, Entwisle & Foard 1999) or in laboratory culture (Necchi Júnior & Carmona 2002).

Populations of *Thorea* have been relatively well-documented in Brazil. Necchi Júnior (1987) described in detail the vegetative and reproductive features *T. bachmannii*. Necchi Júnior & Zucchi (1997) followed the monographic treatment by Sheath *et al*. (1993) and regarded *T. bachmannii* as a synonym of *T. violacea*, and presented a full description, as well as environmental and geographical information. Necchi Júnior *et al.* (1999) reported *T. violacea* from hard water regions in São Paulo State, including environmental data. More recently, Carmona & Necchi Júnior (2001) analyzed four populations from southeastern Brazil, together with others from Central Mexico. They regarded *T. violacea* as a synonym of *T. hispida*, since the most distinguishing character to separate them (secondary branching frequency) showed a considerable overlapping within a same population.

Considering the relatively poor knowledge on stability of taxonomic characters and distributional aspects for *Thorea* species worldwide, this study aimed to evaluate species level taxonomy and phylogenetic relationship among *Thorea* species in Brazil and other regions of the world using two genes – the plastid *rbc*L and the nuclear SSU rDNA.

Materials and methods

Three samples of *Thorea* were analyzed from two out of the three regions where *Thorea* specimens had been reported from Brazil (Necchi Júnior 1987, 1989, Necchi Júnior & Zucchi 1997, Carmona & Necchi Júnior 2001) (table 1). An additional sample from Dominican Republic (from Culture Collection of Algae and Protozoa, CCAP, Oban, Scotland, UK) was also sequenced for comparison. Voucher specimens preserved in 4% formaldehyde were lodged at SJRP Herbarium (Holmgren & Holmgren 1998). Fresh thalli were desiccated in silica gel and later kept frozen at -20 °C. These samples were ground in liquid nitrogen and DNA was extracted using the NucleoSpin® Plant mini kit (MN – Macherey-Nagel, Düren, Germany) following the manufacturer's protocol.

Polymerase chain reactions (PCR) for both molecular markers were conducted with the "puReTaq Ready-to-go PCR beads" (GE Healthcare Life Sciences, Bucks, UK) for a total volume of 25 µL consisting of 2 µL of genomic DNA, 2 µL of each primer, 19 µL of Nuclease-free water. PCR reactions were performed in a Techne TCS-312 thermocycler (Techne, Cambridge, UK). The genes *rbc*L and SSU rDNA were amplified using primers and cycles previously described (Vis *et al.* 1998, Vis & Sheath 1999, Milstein & Oliveira 2005). PCR product was purified using the QIAquick® kit (Qiagen) according to the manufacturer's protocol.

The double-stranded PCR products were sequenced using the ABI PRISM® Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit in the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, U.S.A.). Sequencing reactions were performed using PCR amplification primers and internal primers (as listed in Vis *et al.* 1998, Vis & Sheath 1999, Milstein & Oliveira 2005) so that the entire fragment was sequenced in both directions. Consensus sequences were obtained for the *rbc*L and SSU rDNA genes for the samples listed in table 1, encompassing 1,282 bp for *rbc*L and 1,752 bp for SSU rDNA. For sample BO 8, the SSU rDNA sequence was 1,565 bp. Unfortunately, we were not able to get PCR products from samples BO 8

Table 1. Sample information and GenBank accession numbers for *rbc*L and SSU rDNA sequences of *Thorea* samples sequenced in this study.

Sample code[1]	Collection information	GenBank accession number[2]
SP 64	São Paulo State: Jumirim, 2 km from town, tributary of Sorocaba River, 23°05'54" S, 47°47'57" W, culture isolate	GU953247 GU953243
BO 5	Mato Grosso do Sul State: Bodoquena, Campina Stream, 20°25'06" S, 56°43'01" W, coll. *O. Necchi Júnior*, 30-V-2002	GU953248 GU953244
BO 8	Mato Grosso do Sul State: Bonito, Jenipapo Stream, 20°59'07" S, 56°27'08" W, coll. *O. Necchi Júnior*, 01-VI-2002	– GU953245
CCAP 1394/4	Dominican Republic: San Cristobal, spring, 20°18'00" N, 49°46'02" W, 1968, culture isolate	– GU953246

[1] Codes as used in figures 1-2. [2] Numbers refer to *rbc*L and SSU rDNA, respectively.

and CCAP 1394/4 (table 1). The consensus sequences were aligned using ClustalW in BioEdit 6.0 software (Hall 1999) and were manually inspected, including sequences from GenBank. *Ballia callitricha* (C. Agardh) Kützing (AF236790, AF149029) was used as outgroup in all analysis. The final alignment matrices contained 50 sequences belonging to 43 taxa and 1,202 positions for *rbc*L and 27 sequences belonging to 20 taxa and 1,630 positions for SSU rDNA.

Phylogenetic analyses were performed with PAUP 4.0b8 (Swofford 2000) and MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The appropriate evolution model was selected using MrModeltest 2.2 (Nylander 2004) under the Akaike Information Criterion (AIC). For the Bayesian analysis two runs of four Markov chains over 4,000,000 generations sampling every 100 generations was employed. The initial 50,000 generations were discarded as burning. A neighbor-joining (NJ) tree (Saitou & Nei 1987) was built with Tamura & Nei (1993) substitution model. A maximum parsimony (MP) tree was inferred by heuristic search, with starting trees obtained by stepwise addition, with random sequence addition (10 replicates) using the tree bisection-reconnection (TBR) branch-swapping algorithm. In both NJ and MP trees, gaps were treated as missing data and all sites were weighted equally. Bootstrap analyses (Felsenstein 1985) were performed with 2,000 replicates for the methods described above. Maximum likelihood (ML) analysis was performed with heuristic search using TBR algorithm, with starting trees obtained via stepwise addition as described for the MP tree. Bootstrap re-sampling was done for 100 replicates. For all analyses, support values were interpreted as follows (for bootstrap and posterior probability, respectively): low (< 70% or 0.70), moderate (71-90%, 0.71-0.90) and high (> 90% or 0.90). For the *rbc*L data set ML analyses were performed using GTR distance model with the same parameters as the NJ analysis estimated from Modeltest: base frequencies A = 0.3732, C = 0.1053, G = 0.1777 and T = 0.3439, rate matrix A-C = 4.5567, A-G = 5.8084, A-T = 1.5908, C-G = 1.6710, C-T = 26.3251 and G-T = 1.0000, proportion of invariable sites = 0.5299, gamma distribution = 0.9782. For SSU data set the following parameters were used: base frequencies A = 0.2538, C = 0.2088, G = 0.2878 and T = 0.2497, rate matrix A-C = 1.0443, A-G = 2.6035, A-T = 1.0310, C-G = 1.0731, C-T = 4.6944, G-T = 1.0000, proportion of invariable sites = 0.6116, and gamma distribution = 0.7557.

Results

Analyses based on *rbc*L sequences – Analyses based on partial sequences (tables 1-2, figure 1) showed that Thoreales formed a distinct monophyletic clade from the Batrachospermales. The clade for the Thoreales had two major branches with high support, representing the genera *Thorea* and *Nemalionopsis* (figure 1). *Thorea* clade had four main branches with high support based on the four methods of analyses, each one representing species: 1) *T. gaudichaudii* with three samples from Asia (Japan and Philippines); 2) *T. violacea*, with four samples from Asia (Japan) and North America (U.S.A. and Dominican Republic, including one sample of *T. riekei*); 3) *T. hispida*, with six samples from Europe (England) and Asia (Japan, including two samples of *T. okadae* and one as *T. violacea*); this clade had moderate support (86%) for ML but high for the other three analyses; 4) a distinct clade with the two Brazilian samples. Minor clades within species also had high support, except for the two samples of *T. gaudichaudii* (AB159650 e AB159651), with moderate support. The sequences for the two Brazilian samples exhibited high identity (97.2%, 1,246 bp). The interspecific variation between the Brazilian samples and the closely related species *T. hispida* ranged from 83.5 to 91.3%, 1,070-1,170 bp (table 2). Thus, and the Brazilian samples were regarded as a single and separate species.

Table 2. Pairwise comparison of *Thorea* species showing *rbc*L uncorrected-p percent similarity (lower left matrix) and nucleotide identity (upper matrix) for Thoreaceae data used in the analyses. New sequences from this study are in boldface. Sequences from GenBank are listed in alphabetical order with their respective accession numbers and species names as reported by the original authors.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Nemalionopsis shawii AB159658-1	–	1200	1205	1106	1104	1103	1123	1124	1114	1113	1041	1104	1108	1108	1063	1051	1038	1104	1111
N. shawii AF506266-2	93.6	–	1128	1037	1036	1033	1051	1051	1041	1041	1110	1032	1038	1036	1135	1122	1109	1031	1037
N. tortuosa AB159659-3	94.0	88.0	–	1105	1104	1101	1127	1127	1122	1120	1045	1091	1095	1095	1059	1050	1023	1111	1118
Thorea gaudichaudii AB159649-4	86.3	80.9	86.2	–	1273	1270	1126	1124	1126	1127	1051	1170	1165	1172	1064	1051	1088	1109	1126
T. gaudichaudii AB159650-5	86.1	80.8	86.1	99.3	–	1274	1123	1122	1123	1124	1050	1169	1164	1170	1065	1050	1087	1111	1127
T. gaudichaudii AB159651-6	86.0	80.6	85.9	99.1	99.4	–	1123	1122	1123	1124	1050	1168	1163	1168	1064	1050	1086	1109	1123
T. okadae AB159654-7	87.6	82.0	87.9	87.8	87.6	87.6	–	1281	1246	1245	1165	1131	1131	1133	1136	1200	1055	1149	1160
T. okadae AB159655-8	87.7	82.0	87.9	87.7	87.5	87.5	99.9	–	1245	1245	1165	1129	1131	1132	1136	1200	1055	1147	1159
T. hispida AB159652-9	86.9	81.2	87.5	87.8	87.6	87.6	97.2	97.1	–	1281	1200	1137	1133	1141	1136	1167	1059	1160	1170
T. hispida AB159653-10	86.8	81.2	87.4	87.9	87.7	87.7	97.1	97.1	99.9	–	1200	1138	1135	1141	1135	1165	1059	1159	1169
T. hispida AF506270-11	81.2	86.6	81.5	82.0	81.9	81.9	90.9	90.9	93.6	93.6	–	1063	1059	1065	1211	1244	1131	1081	1091
T. riekei AB159656-12	86.1	80.5	85.1	91.3	91.2	91.1	88.2	88.1	88.7	88.8	82.9	–	1213	1273	1069	1055	1136	1115	1131
T. violacea AB159657-13	86.4	81.0	85.4	90.9	90.8	90.7	88.2	88.2	88.4	88.5	82.6	94.6	–	1209	1068	1055	1200	1109	1123
T. violacea AF029160-14	86.4	80.8	85.4	91.4	91.3	91.1	88.4	88.3	89.0	89.0	83.1	99.3	94.3	–	1072	1059	1133	1117	1131
T. violacea AF506268-15	82.9	88.5	82.6	83.0	83.1	83.0	88.6	88.6	88.6	88.5	94.5	83.4	83.3	83.6	–	1213	1141	1083	1097
T. violacea AF506269-16	82.0	87.5	82.1	82.0	81.9	81.9	93.6	93.6	91.0	90.9	97.0	82.3	82.3	82.6	94.6	–	1127	1070	1082
T. violacea AF506271-17	81.0	86.5	79.8	84.9	84.8	84.7	82.3	82.3	82.6	82.6	88.2	88.6	93.6	88.4	89.0	87.9	–	1036	1050
***T. bachmannii* SP 64-18**	86.1	80.4	86.7	86.5	86.7	86.5	89.6	89.5	90.5	90.4	84.3	87.0	86.5	87.1	84.5	83.5	80.8	–	1246
***T. bachmannii* BO 5-19**	86.7	80.9	87.2	87.8	87.9	87.6	90.5	90.4	91.3	91.2	85.1	88.2	87.6	88.2	85.6	84.4	81.9	97.2	–

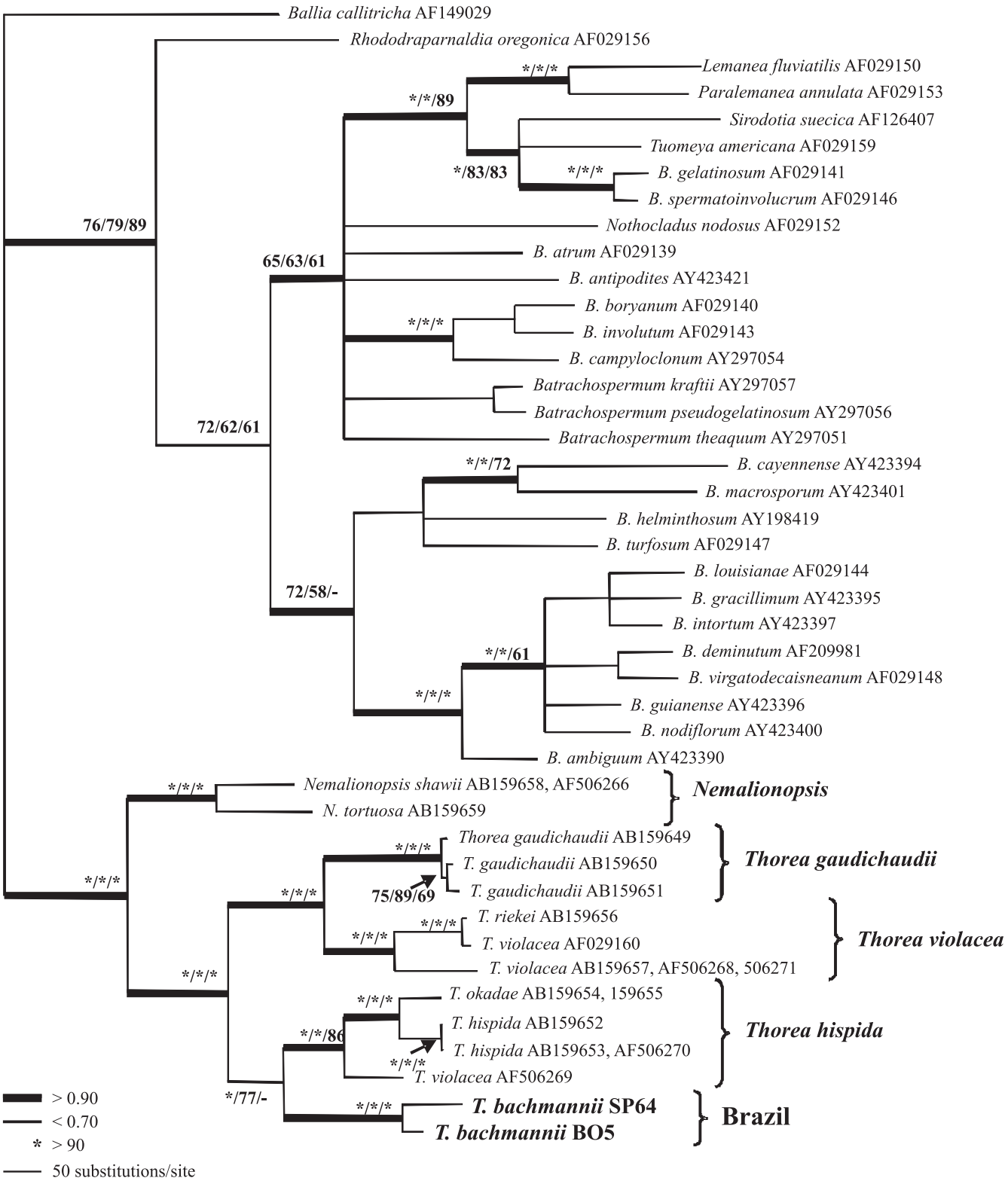

Figure 1. Maximum likelihood (ML) *rbc*L analysis tree showing the relationships of the samples from Brazil (larger and boldface) to previously sequenced taxa of Thoreales. Bootstrap support values for all analyses are shown on the branches as follows: neighbor-joining (NJ)/Maximum Parsimony (MP)/Maximum likelihood (ML) bootstraps. Bayesian posterior probabilities are shown as branches with distinct thickness (as indicated in the figure).

Analyses based on SSU rDNA sequences – Analyses (tables 1, 3, figure 2) revealed that Thoreales formed a monophyletic clade apart from the Batrachospermales and within the Thoreales two major clades were formed with high support, representing the genera *Thorea* and *Nemalionopsis* (figure 2). *Thorea* clade had two main branches with high support, one representing *T. violacea* and the other included two closely related species (*T. hispida* and the Brazilian samples). The clade of *T. violacea* had four samples from North America (U.S.A., including the new sequence from Dominican Republic). The clade of *T. hispida* included three samples from Asia (Australia and Japan, as *Thorea* sp. and *T. violacea*, respectively) and Europe (England). The third clade included the three Brazilian samples. The sequences for Brazilian samples exhibited higher identity with one another (96.0-98.1%, 1,699-1,720 bp) than to the closer related species *T. hispida* (90.5-95.5%, 1,586-1,674 bp, table 3). In addition, this variation among sequences of the Brazilian samples was comparable to those observed within the other two species: *T. hispida* (95.4-99.3%, 1,672-1739 bp) and *T. violacea* (95.3-99.3%, 1,671-1,740 bp). Thus, the samples from Brazil were interpreted as representing a single and distinct species.

Table 3. Pairwise comparison of *Thorea* species showing SSU rDNA uncorrected-p percent similarity (lower left matrix) and nucleotide identity (upper matrix) for Thoreaceae data used in the analyses. New sequences from this study are in boldface. Sequences from GenBank are listed in alphabetical order with their respective accession numbers and species names as reported by the original authors.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12
Nemalionopsis tortuosa AF342743 – 1	–	1714	1604	1697	1704	1595	1639	1671	1697	1625	1639	1629
N. shawi AF506272 – 2	97.8	–	1620	1681	1686	1611	1653	1651	1672	1602	1616	1611
T. hispida AF506273 – 3	91.5	92.4	–	1615	1618	1739	1674	1685	1613	1595	1613	1622
T. violacea AF342744 – 4	96.8	95.9	92.1	–	1740	1604	1681	1679	1727	1630	1641	1630
T. violacea AF026042 – 5	97.2	96.2	92.3	99.3	–	1608	1683	1686	1730	1639	1650	1637
T. violacea AF506274 – 6	91.0	91.9	99.2	91.5	91.7	–	1664	1672	1602	1586	1604	1629
T. violacea AF506275 – 7	93.5	94.3	95.5	95.9	96.0	94.9	–	1622	1671	1574	1588	1590
Thorea sp. AF420253 – 8	95.3	94.2	96.1	95.8	96.2	95.4	92.5	–	1342	1662	1674	1660
***Thorea violacea* CCAP 1394/4 – 9**	96.8	95.4	92.0	98.5	98.7	91.4	95.3	95.7	–	1634	1643	1637
***Thorea bachmannii* SP 64 – 10**	92.7	91.4	91.0	93.0	93.5	90.5	89.8	94.8	93.2	–	1699	1683
***Thorea bachmannii* BO 5 – 11**	93.5	92.2	92.0	93.6	94.1	91.5	90.6	95.5	93.7	96.9	–	1720
***Thorea bachmannii* BO 8 – 12**	92.9	91.9	92.5	93.0	93.4	92.9	90.7	94.7	93.4	96.0	98.1	–

Discussion

In previous phylogenetic analyses of the Thoreales (Vis *et al.* 1998, Müller *et al.* 2002) it consistently appeared as well-supported clade, as well as the two genera (*Thorea* and *Nemalionopsis*). Likewise, in the present study, the order and its two genera were confirmed and further supported with the addition of two new *rbc*L and four new SSU rDNA sequences (three from Brazil and one from Dominican Republic). Thoreales is distinguished from members of the sister group Batrachospermales by having multiaxial thallus construction (Sheath *et al.* 1993, Kumano 2002). The two genera in the Thoreales can be separated on reliable morphological characters: *Thorea* has reproductive structures (carpogonia, spermatangia, carposporangia and monosporangia) positioned at the base of assimilatory filaments that are not contained in a common gelatinous matrix, whereas in *Nemalionopsis* reproductive structures are at the apex of assimilatory filaments, which are embedded in a gelatinous matrix (Sheath *et al.* 1993, Müller *et al.* 2002).

Thus, the available morphological and molecular information indicates that the Thoreales is well-resolved at ordinal and generic levels.

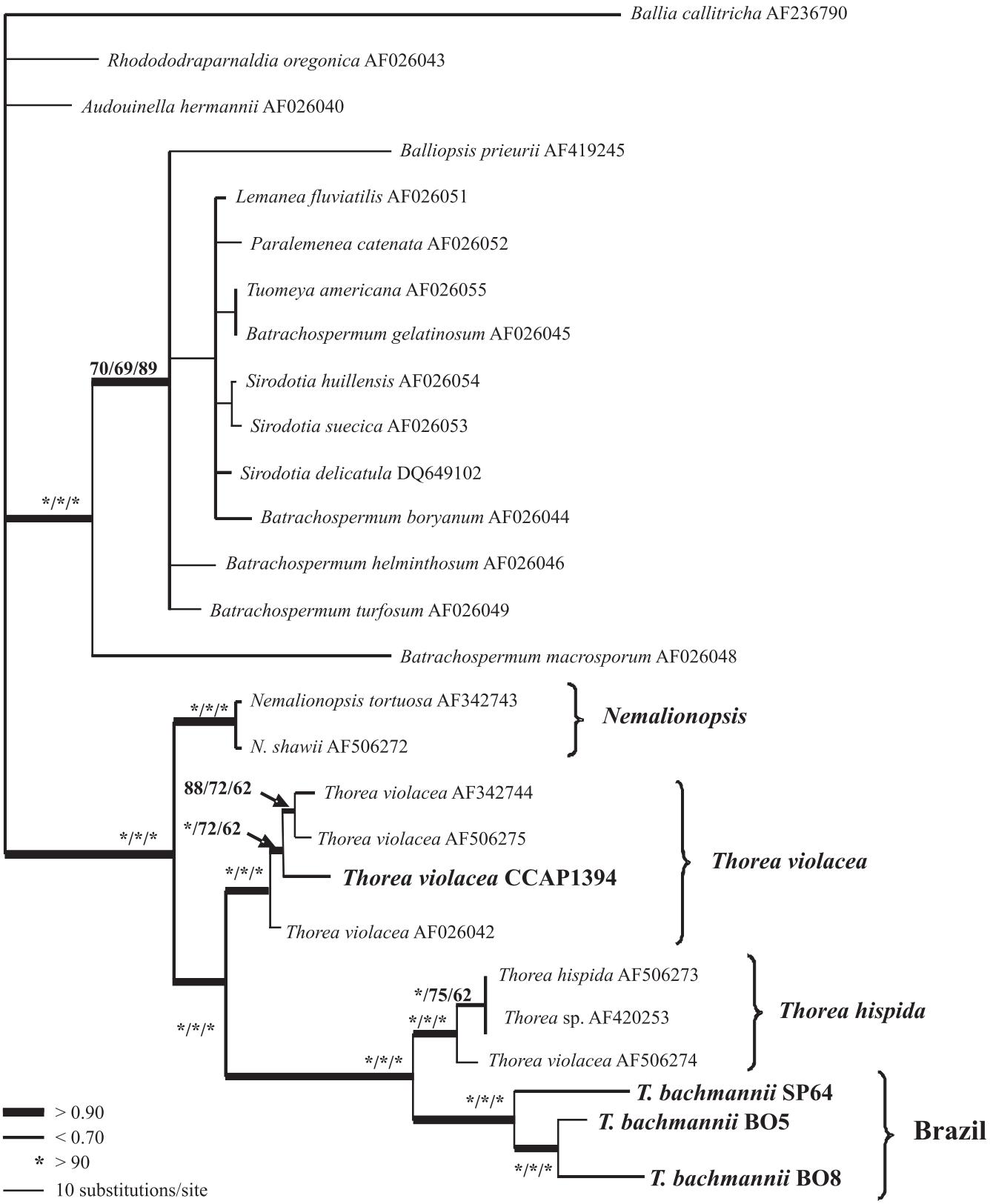

Figure 2. Maximum likelihood (ML) SSU rDNA analysis tree showing the relationships of the samples from Brazil (larger and boldface) to previously sequenced taxa of Thoreales. Bootstrap support values for all analyses are shown on the branches as follows: neighbor-joining (NJ)/Maximum Parsimony (MP)/Maximum likelihood (ML) bootstraps. Bayesian posterior probabilities are shown as branches with distinct thickness (as indicated in the figure).

Molecular data based on the two markers used in this study were quite consistent and congruent at species level. The dataset for *rbc*L covers a higher number of species and populations of each species and thus it is more representative. Four species can be recognized on the basis of molecular data: 1) *T. gaudichaudii* – from Asia (Japan and Philippines, T. Hanyuda *et al.*, unpublished data); 2) *T. violacea* – from Asia (Japan) and North America (U.S.A. and Dominican Republic) (T. Hanyuda *et al.*, unpublished data, Müller *et al.* 2002, this study); 3) *T. hispida* – from Asia (Australia and Japan) and Europe (England) (T. Hanyuda *et al.*, unpublished data, Müller *et al.* 2002, Saunders & Necchi Júnior 2002); 4) the three Brazilian populations formed a highly supported and distinct clade. According to the taxonomic account by Sheath *et al.* (1993) based on morphology, we confirmed that *T. riekei* did not appear as a distinct entity, and should be considered as a synonym with *T. violacea*. However, samples of *T. okadae* from Japan (T. Hanyuda *et al.*, unpublished data) did not form a separate branch, but grouped together with samples from Japan and England (Müller *et al.* 2002) and should be treated as a synonym of *T. hispida* and not of *T. violacea* as proposed by Sheath *et al.* (1993). *Thorea violacea* was considered as paraphyletic by Müller *et al.* (2002) because one sample (sequences AF506269 – *rbc*L and AF506274 – SSU) was associated with *T. hispida*, a result confirmed in this study. However, it would be presumably a case of misidentification of that particular sample, since all other populations of *T. hispida* and *T. violacea* grouped with their allies, as expected, including our new SSU sequence of *T. violacea* from Dominican Republic. The species here delineated partly agrees with the worldwide species treatment adopted by Kumano (2002) by recognition of *T. gaudichaudii*, *T. hispida* and *T. violacea* as distinct species. However, it differs in that present molecular data did not support the acceptance of *T. okadae* and *T. riekei* as distinct taxonomic entities at any rank (specific or infra-specific level).

The Brazilian samples clearly formed a distinct monophyletic clade based on both molecular markers and is here interpreted as a separate species. In the first unequivocal report of *Thorea* in Brazil (Necchi Júnior 1987, 1989) Brazilian specimens were identified as *T. bachmannii*, a species originally described from Argentina (Pujals 1967). The species was later considered as a synonym with *T. violacea* (Sheath *et al.* 1993), which has been followed by Necchi Júnior & Zucchi (1997). *Thorea bachmannii* appeared as a sister group of *T. hispida*, with which it has been previously considered as a synonym (Carmona & Necchi Júnior 2001). However, it is not closely related to *T. violacea* on the basis of *rbc*L and SSU sequences, as it was previously assumed by Sheath *et al.* (1993) and Necchi Júnior & Zucchi (1997) on a morphological ground. Kumano (2002) kept *T. bachmannii* as a separate species from *T. hispida* and *T. violacea*, which has good support from present molecular data. Thus, we resurrected the name *T. bachmannii* for the Brazilian populations as originally reported for specimens from São Paulo State (Necchi Júnior 1987, 1989).

A comparative study of other samples and species from across the world is expected to yield additional tools to separate species and to complement the DNA sequence data presented in this investigation. Considering that wide morphological and morphometrical variations have been reported for vegetative characters (Sheath *et al.* 1993, Carmona & Necchi Júnior 2001, Kumano 2002), species description should include reproductive characters (carpogonia, spermatangia and carposporangia), which could serve as additional diagnostic characters (Yoshizaki 1986, Necchi Júnior 1987, Carmona & Necchi Júnior 2001). Other features can be potentially used as well, *e.g.* chromosome numbers (Carmona & Necchi Júnior 2001). A broad set of characters will possibly contribute towards more consistent and reliable species circumscriptions in *Thorea*. This will be a necessary step to accomplish a world revision of the genus and to test the classification scheme here adopted.

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