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Phylogenetic relationship of *Sirodotia* species (Batrachospermales, Rhodophyta) in North and South America

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Abstract – Sequence data from the RUBISCO large subunit (*rbc*L) plastid gene and nuclear small-subunit ribosomal DNA (SSU rDNA) were examined for five samples of *Sirodotia delicatula* from southeastern Brazil. Data from six North American samples, previously identified as *S. huillensis* and *S. suecica* were also included in the analysis. Molecular data supported the continued recognition of these three species as separate entities, although one of the North American collections was misidentified. These results were shown to be congruent with morphology, chromosome number and geographic distribution. *S. delicatula* is more closely related to *S. huillensis*, both occurring in tropical-subtropical regions, than either to *S. suecica* with a temperate-boreal distribution. There was little *rbc*L variation within *S. delicatula* from Brazil and Costa Rica (the latter a collection previously identified as *S. huillensis*), with the six samples sequenced diverging from each other by 0-8 bp (0-0.67%). SSU rDNA data set did not provide sufficient resolution to infer phylogenetic relationships among the species of this group due to the low rates of variation (5 bp). *Sirodotia* was a well-supported clade (100% bootstrap or 1.00 *a posteriori* probability) based on *rbc*L sequences. Thus, the results confirm that *Sirodotia* is a monophyletic group within the Batrachospermales and we continue to recognize it at the generic level. The species *S. delicatula, S. huillensis* and *S. suecica* are morphologically and genetically distinct.

Batrachospermales / Brazil / freshwater Rhodophyta / *rbc*L gene / *Sirodotia* / SSU rDNA

Résumé – **Relations phylogénétiques des espèces de *Sirodotia* (Batrachospermales, Rhodophyta) en Amérique du Nord et du Sud**. Les séquences de la grande sous-unité de la RUBISCO (*rbc*L), gène plastidial, et de la petite sous-unité de l'ADN ribosomique nucléaire (SSU rDNA) ont été examinées chez cinq échantillons de *Sirodotia delicatula* du sud-est du Brésil. Les données d'échantillons d'Amérique du Nord précédemment identifiés comme *S. suecica* et *S. huillensis* ont été aussi incluses dans l'analyse. Les données moléculaires confirment la reconnaissance de ces trois espèces comme des entités séparées,

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bien que l'une des récoltes nord-américaines ait été mal identifiée. Ces résultats concordent avec les données de la morphologie, les nombres chromosomiques et la distribution géographique. *S. delicatula* est plus proche de *S. huillensis*, ces deux espèces existant dans les régions tropicales et subtropicales, que de *S. suecica*, espèce des régions tempérées et boréales. On note peu de variation du rbcL chez les *S. delicatula* du Brésil et de Costa Rica (cette dernière récolte ayant été précédemment identifiée comme *S. huillensis*), avec les six échantillons séquencés divergeant les uns des autres de 0 à 8 bp (0-0,67%). Les données du SSU rDNA ne fournissent pas une résolution suffisante pour mettre en évidence des relations phylogénétiques parmi les espèces de ce groupe à cause du faible niveau de variation (5 bp). *Sirodotia* est un groupe bien établi (100 % bootstrap ou 1.00 de probabilité à posteriori) sur la base des séquences rbcL. Ainsi, nos résultats confirment que *Sirodotia* est un groupe monophylétique parmi les Batrachospermales et les auteurs continuent à le reconnaître au niveau de genre. Les espèces *S. delicatula*, *S. huillensis* et *S. suecica* sont distinctes sur le plan moléculaire comme sur le plan de la morphologie.

Batrachospermales / Brésil / gène *rbc*L / Rhodophytes dulçaquicoles / *Sirodotia* / SSU rDNA

INTRODUCTION

The genus *Sirodotia* was proposed by Kylin (1912) within the family Batrachospermaceae. Necchi & Entwisle (1990) proposed that it be reduced to a section of the genus *Batrachospermum* based on the fact that the main distinguishing character (presence of indeterminate gonimoblast filaments) is also found in most members of section *Turfosa*, and its recognition at genus level would make *Batrachospermum* paraphyletic. This proposal has been adopted in some taxonomic accounts of the group (e.g. Necchi, 1991), but not in others (e.g. Necchi *et al*., 1993; Entwisle & Foard, 1999). Kumano (1982) described six species from Japan and Malaysia, including two new species (*S. segawae* Kumano and *S. yutakae* Kumano), both having specialized spermatangial branches. Necchi *et al.* (1993) examined 25 samples and 10 type specimens and recognized three species in North America: the type species *S. suecica* Kylin, *S. huillensis* W. West *et* G.S. West and *S. tenuissima* Flint. Vis & Sheath (1999) proposed that *S. tenuissima* be synonymized with *S. suecica* based on similarities of DNA sequences of RUBISCO large subunit (*rbc*L) gene and *rbc*L-S spacer. They concluded that the morphological characters (primarily size) used to distinguish them were not phylogenetically informative. Entwisle & Foard (1999) recognized two species from Australasia: the widespread *S. suecica* and a new species, *S. goebeli* Entwisle *et* Foard. Kumano (2002) recognized eight species worldwide.

Sirodotia has been relatively well-studied in Brazil, both taxonomically (Necchi, 1991) and ecologically (Pascoaloto & Necchi, 1990; Necchi, 1993, Necchi *et al*., 1999, Necchi & Branco, 1999). *Sirodotia delicatula* Skuja (syn. *Batrachospermum delicatulum* (Skuja) Necchi *et* Entwisle) is the only species recognized in Brazil and one of the most widespread species of freshwater red algae in the country (Necchi, 1991; Necchi *et al*., 1999). However, the phylogenetic relationship of this species with its North American counterparts has never been investigated, including an evaluation if it represents a separate entity in the genus. In addition, the systematic status of *Sirodotia* has not been adequately evaluated, that is, if it should be kept as a separate genus or as a section within the genus *Batrachospermum*. Phylogenetic molecular investigations of North American

species of *Sirodotia* including representative members of the Batrachospermales using *rbc*L and the nuclear encoded small-subunit ribosomal (SSU rDNA) gene sequences (Vis *et al*., 1998; Vis & Sheath, 1999) revealed that it consistently appeared as a monophyletic group. One *S. suecica* sample from Australia with partial (723 bp) *rbc*L sequence data grouped with North American representatives of this species and *S. huillensis* (Vis & Entwisle, 2000). Vis *et al.* (1998) recommended that *Sirodotia*, together with other genera of the Batrachospermaceae (*Nothocladus* and *Tuomeya*), continue to be recognized at the generic level until further research on a wider sampling of specimens could be performed.

This study was undertaken to compare *Sirodotia* samples from Brazil to those of the genus *Sirodotia* previously studied in North America, based on two molecular markers (*rbc*L and SSU rDNA genes). The entities recognizable by the molecular analysis are related to the relevant taxonomic information available for *Sirodotia* species in North and South America.

MATERIAL AND METHODS

Five samples of *S. delicatula* were analyzed from southeastern Brazil (Table 1). Samples from distinct locations were used in order to survey within species molecular variation (Table 1). Voucher specimens preserved in 4% formaldehyde were lodged at Herbarium SJRP (Holmgren & Holmgren, 1993). 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

Table 1. Sample information and GenBank accession numbers for *rbc*L and SSU rDNA sequenced for collections of *Sirodotia* samples from Brazil.

Sirodotia delicatula sample code[1]	*Collection information*	*GenBank Accession Number*[2]
C3	Minas Gerais State: Cipó Mountains National Park, Alto do Palácio Stream, 100 m from Route MG-010, 19°17'00"S, 43°33'00"W, 25.viii.2003; *coll. O. Necchi Jr., L.H.Z. Branco & E.N.M. Moura*	DQ646474 DQ649102
23	São Paulo State: São José do RioPreto, Lagoa Stream, 20°49'35"S, 49°20'37"W, culture isolate	DQ646475 DQ649105
29	São Paulo State: São José do RioPreto, Macaco Stream, 20°50'31"S, 49°21'07"W, culture isolate	DQ646676 DQ649106
TA	São Paulo State: São José do RioPreto, Talhadinho Stream, 20°43'24"S, 49°18'21"W, 07.x.2004; *coll. O. Necchi Jr.*	DQ646677 DQ649104
AP	São Paulo State: Américo de Campos, Águas Paradas Stream, 20°18'00"S, 49°46'02"W, 06.x.2004; *coll. O. Necchi Jr.*	DQ646678 DQ649103

1. Codes as used in Figure 1.
2. Numbers refer to *rbc*L and SSU rDNA, respectively.

DNeasy® Plant Mini kit (Qiagen, Valencia, California) following the manufacturer's protocol.

Polymerase chain reaction (PCR) for SSU rDNA was conducted with a Promega PCR Master Mix (Promega, Madison, USA) for a total volume of 50 μL consisting of 2.5 μL of genomic DNA, 2.5 μL of each primer, 17.5 μL of Nuclease-free water and 27.5 μL of PCR Mix. PCR reactions were performed in a Techne TCS-312 thermocycler (Techne, Cambridge, UK). PCR amplification for *rbc*L was performed in a Perkin Elmer 2400 thermocycler with a reaction volume of 50 μL and consisted of 2 μL genomic DNA, 10 μM each of dATP, dTTP, dGTP and dCTP, 0.4 μM of each primer, 2 mM $MgCl_2$ and 10X reaction buffer with 1 unit Taq Polymerase (Invitrogen, Carlsbad, California). A portion (1282 bp) of the *rbc*L and the entire SSU rDNA gene (1724 bp) 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 and the ABI PRISM 310 (or 3100) Genetic Analyzer (Applied Biosystems, Foster City, California). 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. SSU rDNA sequences were manually assembled with BioEdit 6.0 software (Hall 1999), whereas *rbc*L sequences were assembled in Sequencher™ version 4.1 (Gene Codes Corp, Ann Arbor, MI).

MacIntosh Genetic Data Environment (MacGDE, version 2.2) was utilized as a platform to run the analyses. Maximum parsimony (MP), maximum likelihood (ML) and minimum evolution (ME) analyses were performed using PAUP* version 4.0b10 (Swofford, 2000). MP trees were generated using a heuristic search under the constraints of random sequence addition (1000 replications) and tree bisection-reconnection (TBR) branch-swapping. The appropriate model of sequence evolution for the data set was determined by the hierarchical likelihood ratio test of goodness of fit for 56 different nested models to the data using the software Modeltest version 3.7 (Posada & Crandall, 1998). ME analysis was conducted using the general time reversible (Rodríguez *et al.*, 1990) distance matrix with gamma distribution = 0.9297, proportion of invariable sites = 0.5214 and a neighbor-joining algorithm. ML analysis was performed using the same parameters as the ME analysis along with other estimated parameters from Modeltest as follows: base frequencies A = 0.3465, C = 0.1142, G = 0.1809 and T = 0.3584 and rate matrix A-C = 3.4741, A-G = 5.0731, A-T = 1.7190, C-G = 2.1773, C-T = 20.7254 and G-T = 1.0000. The most likely tree was constructed from a heuristic search using 100 random additions and TBR swapping. Resulting trees from the MP and ME analyses were subjected to bootstrap analysis as implemented in PAUP* with 10 random additions and 1000 replicates, and those from the ML analysis to bootstrap analysis as implemented in PAUP* with 3 random additions and 100 replicates. Bayesian analysis of the data set was performed using the software program MrBayes version 3.0b4 (Ronquist & Huelsenbeck, 2003). Four Markov chains over 500,000 generations sampling every 100 generations was employed. Posterior probabilities were calculated from the final 4000 trees. For SSU rDNA data (not shown), similar analyses were performed. Distance (neighbor-joining, NJ) analysis was performed using the GTR (Rodríguez *et al.*, 1990). ML analysis was performed using the same parameters from the distance analysis. The estimated

parameters from Modeltest are as follows: base frequencies A = 0.4053, C = 0.1026, G = 0.0974, T = 0.3947 and rate matrix A-C = 1.4710, A-G = 4.8384, A-T = 0.3233, C-G = 2.7395, C-T = 10.4673.

All sequence data generated in this study were submitted to GenBank (Table 1). For analysis of the *rbc*L and SSU rDNA gene data, trees were constructed using previously published batrachospermalean and closely related taxa from GenBank.

RESULTS

*rbc*L sequences

Partial sequences of *rbc*L revealed that *S. delicatula* (including the misidentified Costa Rican collection – see below) differed from *S. huillensis* by 29-34 bp (2.44-3.00%) and *S. suecica* by 60-66 bp (4.76-5.32%); *S. huillensis* differed from *S. suecica* by 66-72 bp (5.30-5.62%) (Table 2). The five Brazilian samples sequenced differed from each other by 0-8 bp (0-0.67%). *Sirodotia* formed a well-supported clade (100% bootstrap or 1.00 *a posteriori* probability) in all trees based on the four methods of analyses (Fig. 1). Samples from each species formed well-supported clades, with high support (98%, 1.00) as follows: *S. delicatula,* containing the five Brazilian samples and one sample from North America (previously reported as *S. huillensis* – AF126410, Table 2), *S. huillensis*, with two samples from North America; and *S. suecica*, with three samples from North America.

Table 2. Pairwise comparison of *Sirodotia* species showing *rbc*L uncorrected-p percent nucleotide difference (lower left matrix) and nucleotide changes (upper matrix). Specimen designations either by Genbank number or as in Table 1.

Taxon	23	AP	TA	29	AF126410	C3	AF029157	AF126414	AF029158	AF126407	AF126420
S. delicatula 23	---	0	0	1	3	8	34	33	62	64	66
S. delicatula AP	0	---	0	1	3	8	34	33	62	64	66
S. delicatula TA	0	0	---	1	3	8	34	33	62	64	66
S. delicatula 29	0.08	0.08	0.08	---	2	7	32	32	61	63	65
S. delicatula AF126410	0	0.23	0.23	0.16	---	6	33	33	62	64	66
S. delicatula C3	0.67	0.67	0.67	0.59	0.5	---	29	29	60	60	63
S. huillensis AF029157	3	2.57	2.57	2.5	2.65	2.44	---	2	68	66	72
S. huillensis AF126414	2.57	2.57	2.57	2.5	2.65	2.44	0.16	---	68	68	72
S. suecica AF029158	4.83	4.83	4.83	4.76	4.84	5.06	5.3	5.3	---	2	4
S. suecica AF126407	5	5	5	4.92	5	5.06	5.31	5.3	0.16	---	6
S. suecica AF126420	5.15	5.15	5.15	5.07	5.15	5.32	5.62	5.62	0.31	0.47	---

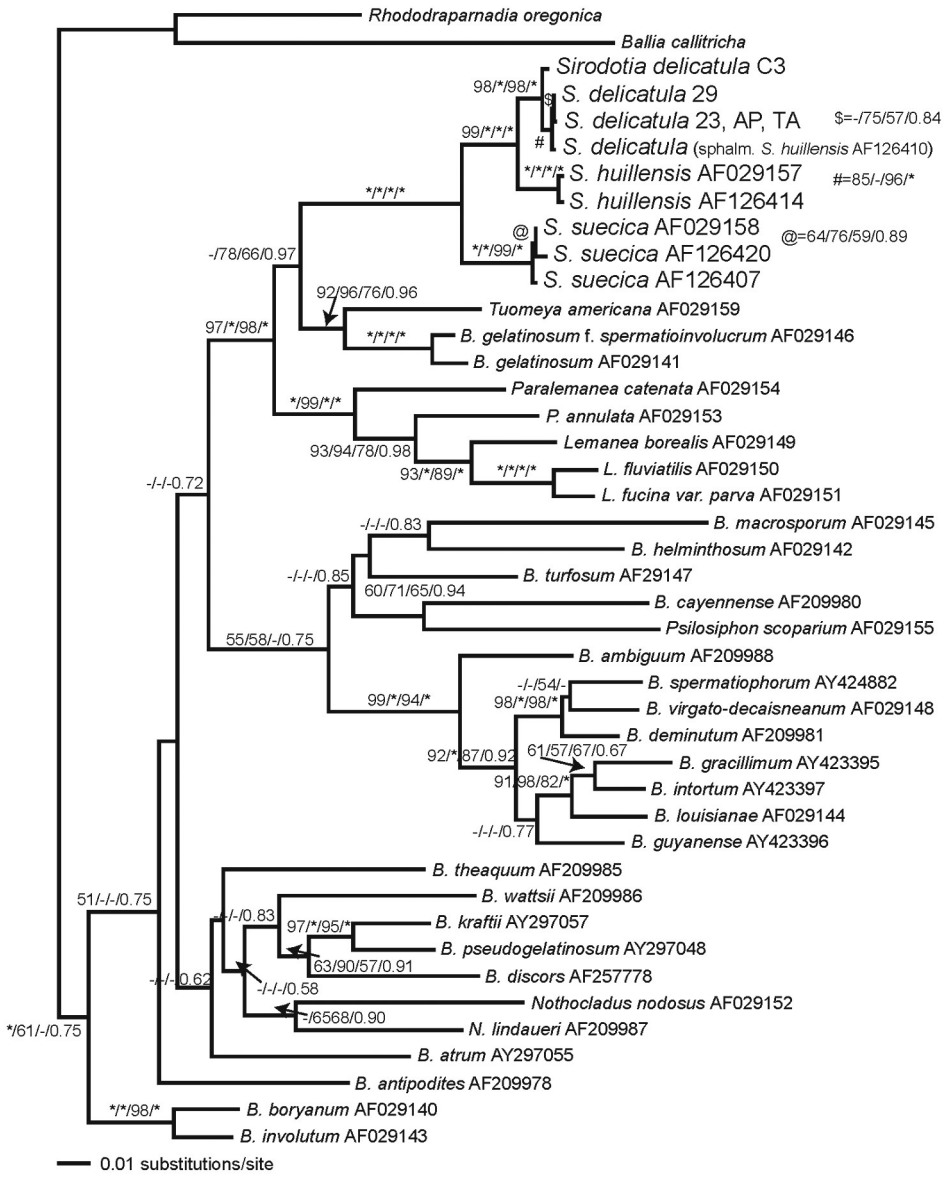

Fig. 1. Maximum likelihood (ML) *rbc*L analysis tree (-ln likelihood = 15451.26101) showing the relationships of the *Sirodotia* samples (larger type) to previously sequenced batrachospermalean taxa. Support values for all analyses are shown on the branches as follows: MP bootstrap/ME bootstrap/ML bootstrap/Bayesian posterior probabilities. An "*" indicates 100% bootstrap support or 1.00 *a posteriori* probability in all analyses. A "-" indicates less than 50% support or probability.

SSU rDNA sequences

Complete sequences of SSU rDNA showed that *S. delicatula* differed from *S. huillensis* by 3-4 bp (0.18-0.24%) and *S. suecica* by 3-5 bp (0.18-0.30%); *S. huillensis* differed from *S. suecica* by 3 bp (0.18%) (Table 3). The five *S. delicatula* samples sequenced had 0-1 bp (0-0.06%) differences. Due to the very low resolution for SSU rDNA sequences, the tree nodes showing the relationship among the Brazilian samples and with the other two species was not well-defined and the tree is not shown.

Morphological Observations

The voucher specimen for *S. huillensis* from Costa Rica (sample CR 22; mistakenly reported as CR 23 in Vis & Sheath, 1999) was re-examined based on the results of the molecular data, since *rbc*L sequence for that sample (GenBank accession number AF126410) showed a much higher affinity to the samples of *S. delicatula* than to the other samples of *S. huillensis*. This specimen has gonimoblast initial developing from the protuberant side of carpogonium and spermatangia arranged singly or in pairs, and thus is attributable to *S. delicatula* and not *S. huillensis* (Table 4).

Table 3. Pairwise comparison of *Sirodotia* species showing SSU rDNA uncorrected-p percent nucleotide difference (lower left matrix) and nucleotide changes (upper matrix). Specimen designations either by Genbank number or as in Table 1.

Taxon	*23*	*AP*	*TA*	*29*	*C3*	*AF26054*	*AF26053*
S. delicatula 23	–	1	1	1	1	4	5
S. delicatula AP	0.06	–	0	1	1	3	5
S. delicatula TA	0.06	0	–	1	1	3	5
S. delicatula 29	0.06	0.06	0.06	–	1	4	5
S. delicatula C3	0.06	0.06	0.06	0.06	–	4	5
S. huillensis AF26054	0.24	0.18	0.18	0.24	0.24	–	3
S. suecica AF26053	0.30	0.30	0.30	0.30	0.30	0.18	–

Table 4. Summary of taxonomically useful characters for distinguishing the three species of *Sirodotia* recognized from North and South America.

Species	*Characters*				
	Gonimoblast initial origin	*Chromosome number*	*Spermatangia arrangement*	*Geographic distribuion in the Americas (Figs 2-3)*	*References*
Sirodotia delicatula	Protuberant side of carpogonium	n=3, 2n=6	singly or in pairs	South and southern North America; widespread in tropical and subtropical regions	Necchi (1991), Necchi & Sheath (1992), Necchi & Carmona (2002)
Sirodotia huillensis	Protuberant side of carpogonium	n=4, 2n=8	dense clusters	North America Desert-Chaparral, common in dry regions	Necchi *et al.* (1993), Carmona *et al.* (2006)
Sirodotia suecica	Non-protuberant side of carpogonium	n=3, 2n=6	singly or in pairs	North America widespread in temperate and boreal regions	Necchi *et al.* (1993), Kumano (1979, 1982)

DISCUSSION

Divergence rates among *rbc*L sequences for *S. delicatula* were similar to those from a previous study of other species of *Sirodotia* (Vis & Sheath, 1999). Samples of *S. huillensis* and *S. suecica* from North America had two to six base pair differences (0.16-0.47%, Vis & Sheath, 1999). In contrast, within species variation of *rbc*L sequences for *Sirodotia* species were considerably lower than those reported for *Batrachospermum helminthosum* Bory from Japan (Hanyuda *et al.*, 2004) and from North America (Vis *et al*., 2001), which revealed divergences of 2.3-4.2% and 0.01-1.4%, respectively. The SSU rDNA data set did not provide sufficient resolution to infer phylogenetic relationships among the species of this group due to the low rates of variation.

In previous analyses of the Batrachospermales, the species of *Sirodotia* have formed a well-supported clade (Vis *et al.*, 1998; Vis & Sheath, 1999). Likewise, in the present study, this group is well supported with the addition of five new *rbc*L sequences from Brazilian samples of *S. delicatula*. Thus, the available information for *Sirodotia* in the Americas indicates that it is clearly a monophyletic group within the Batrachospermales. However, the placement of *Sirodotia* within the larger clade including other taxa of Batrachospermaceae and the Lemaneaceae is not conclusive. At present we follow the suggestion of Vis *et al.* (1998) that *Sirodotia*, together with other genera of Batrachospermaceae (*Nothocladus* and *Tuomeya*), continue to be recognized at generic level. This decision seems sensible until more molecular and morphological data can provide a better resolution of the phylogenetic relationships within the Batrachospermales. The only sample of *Sirodotia* sequenced outside of North and South America, was a single sample of *S. suecica* from Australia (AF209977), whose partial sequence (723 bp) diverged from North American samples only by 4 bp (Vis & Entwisle, 2000) and grouped with the North American samples of this species.

Molecular data based on the two markers used in this study are consistent with the taxonomic data available (Table 4). The three species from the Americas are easily distinguished and *S. delicatula* is the only species occurring in Brazil. *S. huillensis* had been reported from a few southern localities in North America by Vis & Sheath (1999). However, we have reexamined the southernmost sample from Costa Rica and confirmed that it belongs to *S. delicatula*, corroborating the molecular data. This fact extends the geographic distribution in the Americas of *S. delicatula* (Necchi, 1991) to 10 degrees north in North America.

S. delicatula is similar to *S. huillensis* in terms of post-fertilization morphology (gonimoblast initial developing from the protuberant side of carpogonium), both differing from *S. suecica* (gonimoblast initial developing from the non-protuberant side of carpogonium) (Table 4). *S. huillensis* is unique in its spermatangia arrangement (in dense clusters) in comparison to the other two species (singly or in pairs). *S. huillensis* has also a different chromosome number (haploid and diploid numbers 4 and 8, respectively) than the other two *Sirodotia* species (n=3 and 2n=6). In terms of geographic range, *S. suecica* occurs only in temperate and boreal regions of North America, whereas the other two species have a tropical to subtropical distribution (Figs 2-3). In addition, *S. huillensis* has a more southern and localized distribution in North America, occurring in Mexico and the States of Arizona and Texas, and is apparently restricted to the desert-chaparral biome. Thus, morphology, geographic distribution and

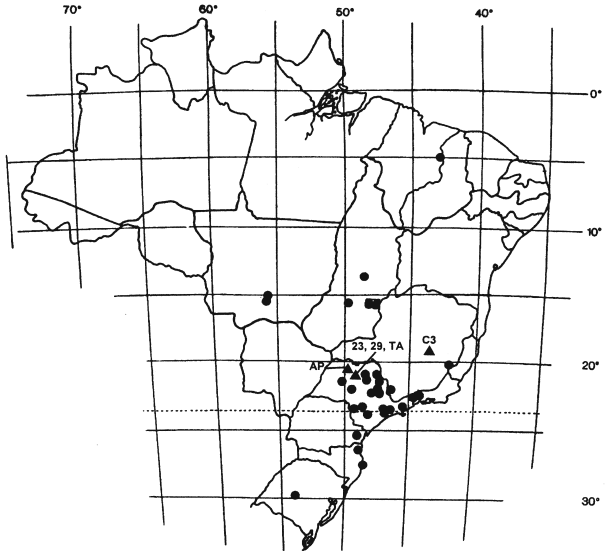

Fig. 2. Distribution of *Sirodotia delicatula* in Brazil: ● - after Necchi (1991); ▲ - samples from this study (see Table 1).

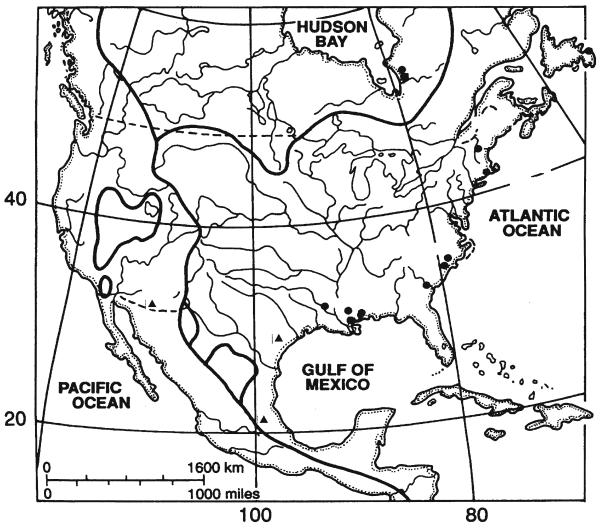

Fig. 3. Distribution of *Sirodotia* in North America: ● *S. suecica* (according to Necchi *et al.* 1993); ▲ *S. huillensis* (according to Necchi *et al.*, 1993; Carmona *et al.*, 2006).

molecular data all correlated and each provides a good basis for distinguishing the three species recognized from the Americas. A comparative study of other samples and species from across the world, including particularly Asian and European representatives, is expected to yield valuable tools to separate species and to complement the DNA sequence data presented in this investigation. This step will be necessary before potential revision to the herein adopted classification scheme is made.

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