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PHYLOGEOGRAPHY OF *BATRACHOSPERMUM MACROSPORUM* (BATRACHOSPERMALES, RHODOPHYTA) FROM NORTH AND SOUTH AMERICA[1]

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Phylogeographic trends in *Batrachospermum macrosporum* Mont. were investigated using the mitochondrial intergenic spacer between the cytochrome oxidase subunit 2 and 3 genes (*cox*2-3). A total of 11 stream segments were sampled with seven in the coastal plain of North America and four in tropical areas of South America. Fifteen thalli were sampled from seven streams, 14 thalli from two streams, and eight thalli from two streams. There were 16 haplotypes detected using 149 individuals. Of the eight haplotypes from locations in North America, all were 334 base pairs (bp) in length, and of those from South America, five were 344 bp, and three were 348 bp. Two individual networks were produced: one for the haplotypes from North America and another for those from South America, and these could not be joined due to the large number of base pair differences. This split between haplotypes from North and South America was confirmed with sequence data of the *rbc*L gene. There was very little genetic variation among the haplotypes from the North American locations, leading us to hypothesize that these are fairly recent colonization events along the coastal plain. In contrast, there was high variation among haplotypes from South America, and it would appear that the Amazon serves as a center of diversity. We detected considerable variation in haplotypes among streams, but frequently, a single haplotype in an individual stream segment, which is consistent with data from previous studies of other batrachospermalean taxa, may suggest a single colonization event per stream.

Key index words: **Brazil; *cox*2-3 spacer; French Guiana; freshwater; *rbc*L gene**

***Abbreviations:* COI, cytochrome oxidase subunit I marker; *cox*2-3, cytochrome oxidase subunit 2 and 3 genes; ISSR, inter-simple sequence repeat; ITS, internal transcribed spacer; *rbc*L, RUBISCO LSU gene**

The freshwater red alga *B. macrosporum* was first described from material collected in tropical streams of French Guiana in northern South America (Montagne 1850). This taxon is one of the largest species of the genus, with gametophyte thallus width up to 2.5 mm and length typically 10–15 cm reaching up to 0.5 m (Necchi 1990, Kumano 2002). This size makes it a very conspicuous macroalga in streams. It can dominate the substratum of small streams and tends to grow on submerged portions of streamside vegetation in large rivers (Necchi 1990, Vis et al. 2004). Although this alga has been collected in streams with rocky bottoms and clear waters, it is more often reported in black-water, sandy-bottomed streams (Whitford and Schumacher 1968, Woodson and Wilson 1973, Necchi 1990, Vis et al. 2004).

B. macrosporum has been collected from numerous locations in both North and South America. In North America, this alga appears to be restricted to streams of the coastal plain and tropical areas. It has been collected in Belize, Cuba, and coastal streams of the southeastern United States, reaching its northern limit in the pine barrens of New Jersey (Sheath et al. 1994 and references therein). In South America, this taxon has only been reported from French Guiana, Bolivia (chantransia stage only), and Brazil (Montagne 1850, Kumano and Necchi 1990, Necchi 1990, Chiasson et al. 2005). However, other parts of South America, which would have streams with similar physical and chemical attributes, have not been well studied for freshwater red algae, and their occurrence is undoubtedly underestimated. In a recent survey of 18 coastal streams in French Guiana, 11 streams had either gametophytes or chantransia stage; likewise, Thérézien (1985) noted it to be ''very common'' in his collections as well. In Brazil, this taxon has a wide distribution from the Amazonas region to coastal streams of the Mata Atlantica in southeastern Brazil and further south (Necchi 1990, Branco and Necchi 1996).

Molecular data for *B. macrosporum* and most members of the Batrachospermales have been limited to studies of phylogenetic relationships (Vis et al. 1998, 2007). There have been only a handful of

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intraspecific genetic variation and phylogeographic studies in the Batrachospermales. Intraspecific variation in the ribosomal internal transcribed spacer (ITS) was studied for a few specimens of *B. gelatinosum* in North America, but this marker provided little variation across a large geographic region (Vis and Sheath 1997). Data from the *cox*2-3 spacer was presented for *B. arcuatum* (as *Chantransia pygmaea*) in the Hawaiian Islands and *Sirodotia delicatula* (as *B. delicatulum*) from southeastern Brazil (Necchi and Vis 2005, Chiasson et al. 2007). Phylogeographic analyses and documentation of intraspecific variation were not the focus of those studies, and the data showed little variation, probably due to the restricted geographic region sampled. Only *B. helminthosum* has been the subject of phylogeographic research and has been well studied using inter-simple sequence repeat (ISSR), ITS, *cox*2-3 spacer, and cytochrome oxidase subunit 1 marker (COI) (Vis et al. 2001, Hall and Vis 2002, Chiasson et al. 2003, House et al. 2008). However, even for this taxon, the phylogeographic research was restricted to eastern North America. There has only been one study that examined sequence data from numerous individuals between two continents. Hanyuda et al. (2004) compared *B. helminthosum* individuals from Japan with those from a previous study in North America using the RUBISCO LSU gene (*rbc*L), which is typically used for phylogenetic systematics rather than phylogeographic studies of a single species. Therefore, the present study of *B. macrosporum* was undertaken to provide phylogeographic data from a second freshwater red algal taxon and present the first data from across two continents using the intraspecific *cox*2-3 marker.

FIG. 1. Map of North and South America showing the sampling locations of *Batrachospermum macrosporum*. For more details regarding locations and abbreviations see Table S1 in the supplementary material.

MATERIALS AND METHODS

Thalli of *B. macrosporum* were collected from 11 streams throughout its range in North and South America in order to study intraspecific genetic variation (Fig. 1). Fifteen thalli were sampled from seven streams, 14 thalli from two streams, and eight thalli from two streams. Details of the collection sites are given in Table S1 (in the supplementary material).

Frozen silica-desiccated samples were ground in liquid nitrogen, and DNA extracted using the Qiagen Plant DNeasy™ (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. PCR amplification was performed in a Cetus 2400 thermocycler (Perkin Elmer, Branchburg, NJ, USA) or an MJ Research Minicycler™ (Bio-Rad, Hercules, CA, USA). The following cycle was used for amplification: initial denaturation at 95°C for 1 min 30 s, 35 cycles at 93°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The primer pair COX2F (5′-GTACCWTCTTTDRGRRKDAAATGTGATGC-3′) and COX3R (5′GGATCTACWAGATGRAAWGGATGT C-3′) was used to amplify the *cox*2-3 spacer region. The primer pair F160 (5′-CCTCAACCAGGAGTAGATCC-3′) and *rbc*LR (5′-CCTCAA CCAGGAGTAGATCCACATTTGCTGTTGGAGTCTC-3′) was used to amplify a 1,282 bp portion of the *rbc*L gene. The PCR products were prepared for sequencing with the Wizard PCR Prep DNA Purification System™ (Promega, Madison, WI, USA) according to the manufacturer's protocol. These products were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems, Foster City, CA, USA) and the ABI 310 Genetic Analyzer. Sequencing reactions were prepared and sequenced with the amplification primers F160 and *rbc*LR and four internal primers for the *rbc*L as follows: R472.1 (5′-CGTTCACGTTCAACTATAAGTCC-3′) or R472.a (5′-CGCTCACGTTCAACTATAAGCCC -3′), F650 (5′-ATTAACTCTCAACCATTTATGCG-3′) or F650.1 (5′-ATTAATTCTCAGCCTTTCATGCG-3′) or F650.wbc1 (5′-ATTAATTCACAGCCATTTATGCG-3′), R897.1 (5′-CGTGAGTATGTTGAATTACCAGC-3′) and F1087.1 (5′-ATCATTTAAGTGTTAATCTACCTC-3′). Sequences were compiled for analysis using Sequencher™ version 4.6 software (Gene Codes, Ann Arbor, MI, USA).

Haplotype genealogies for the *cox*2-3 spacer data were inferred using the cladogram estimation method of Templeton et al. (1992) as implemented in TCS 1.21 (Clement et al. 2000). Analysis of phylogenetic relationships among *rbc*L haplotypes was conducted using PAUP* version 4 (Swofford 2002). Maximum-parsimony (MP) analysis was performed under the following conditions: 100 random sequence additions, tree-bisection-reconnection (TBR) branch swapping, and steepest descent. The appropriate model of sequence evolution for our data set was determined by the hierarchical likelihood ratio test of best fit for 56 models using the software Modeltest 3.7 (Posada and Crandall 1998). Minimum evolution (ME) analysis was performed using the general-time-reversible (GTR) (Rodríguez et al. 1990) distance matrix with gamma distribution = 1.0642, proportion of invariable sites = 0.5528, and neighbor-joining (NJ) algorithm. The ML analysis was conducted using 10 random sequence additions, TBR, and steepest descent conditions and was performed using the same parameters from the ME analysis along with the other estimated parameters from Modeltest as follows: base frequencies A = 0.3526, C = 0.1178, G = 0.1812, and T = 0.3471; and rate matrix A-C = 3.1510, A-G = 5.1039, A-T = 1.1139, C-G = 1.4868, C-T = 17.9783. Resulting trees from the MP and ME were

subjected to bootstrap as implemented in PAUP* with 10 random additions and 1,000 replicates. MrBayes v.3.0b4 (Ronquist and Huelsenbeck 2003) was employed to perform Bayesian analysis using four Markov chains and 500,000 generations, sampling every 100 generations. Posterior probabilities were calculated using the final 4,000 trees. Phylogenetic trees were outgroup-rooted using 20 closely related taxa (Vis et al. 2005). All *rbc*L gene and *cox*2-3 spacer sequence data generated in this study were submitted to GenBank (Table S1).

Voucher specimens for each location are housed at either the University of Michigan herbarium (MICH) or UNESP, Campus São José Rio Prêto (SJRP). All collections were identified prior to sequencing using currently accepted species circumscription and taxonomic characteristics for *B. macrosporum* (Sheath et al. 1994, Kumano 2002). Specimens were reexamined by M. L. V. and O. N. independently after the sequence data were obtained to verify the identity and determine potentially new phylogenetically informative morphological characteristics.

RESULTS

There were 16 *cox*2-3 spacer haplotypes detected using 149 *B. macrosporum* individuals from 11 locations. Haplotypes A–H were 334 bp in length; haplotypes I, J, L, M, and P were 344 bp; and haplotypes K, N, and O were 348 bp. The overall alignment of these sequences was 348 bp. The difference in length among these groups of haplotypes was due to indels near the end of the fragment (bases 296–310). Overall, there was as much as 19.8% sequence variation among haplotypes. Haplotypes A–H varied by only a few substitutions in seven positions and 0.3%–1.8% sequence variation. Likewise, haplotypes K, N, and O varied only at two positions, with 0.3%–0.6% sequence variation. However, among haplotypes I, J, L, M, and P, there were substitutions at 61 positions, with 4.7%–11.9% sequence variation.

Six locations (MS2, 3, AL2, NJ, FG, BR1) had a single haplotype, one location (AL2) had two haplotypes, three locations (MS1, NC, BR3) had three haplotypes, and one location (BR2) had five haplotypes (see Table 1 for explanation of location codes). Haplotype A was the most prevalent in terms of number of locations (5) and individuals (62). In addition to haplotype A, only two other haplotypes (K and M) were shared between two locations, leaving 13 haplotypes exclusive to a single location (Table 1).

The TCS software program provided two networks: one for haplotypes A–H from North America and one for haplotypes I–O from South America (Fig. 2). These two networks could not be joined using a 95% confidence criterion due to the large number of base pair differences (53–74). Haplotype A, which occurred in five of the seven North American locations, was hypothesized to be the ancestral for the eight haplotypes from that continent. Haplotype N only occurred in a single location and was hypothesized to be ancestral for the eight South American haplotypes.

Sequence variation in the *rbc*L gene among haplotypes ranged from 0 to 6% uncorrected p distance. However, only five positions differed among haplotypes A–H, with A, B, and H, and C and D being identical. Likewise, haplotypes K, N, and O were identical, but all others differed by at least one base pair. Phylogenetic analyses (MP, ME, ML, Bayesian) of the *rbc*L gene sequence for each haplotype showed similar topologies such that only the ML tree with bootstrap and posterior probabilities is presented (Fig. 3). The *rbc*L gene data were congruent with the *cox*2-3 spacer data in that haplotypes A–H from North America form a well-supported clade, and haplotypes I–O from South America form another clade.

The morphology of voucher specimens from each location preserved in 2.5% glutaraldehyde was examined and contained thalli with carpogonia within the morphometric range in length and diameter, and trichogyne shape as presented by other authors (Sheath et al. 1994, Kumano 2002). Likewise, all vouchers had the typical large carposporangia. However, vouchers did differ in breeding system as follows: monoecious thalli in

TABLE 1. Geographic origin of the *Batrachospermum macrosporum* samples and absolute frequencies of the haplotypes at each location. Haplotype and site designations as given in Table S1 (see the supplementary material).

		Haplotype															
Site	*n*	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
MS1	15	7	4	4													
MS2	15	15															
MS3	15	15															
AL1	15	11			4												
AL2	14	14															
NC	15					11	3	1									
NJ	15								15								
FG	8									8							
BR1	15										15						
BR2	8											1	1	1	3	2	
BR3	14											1		2			11
Total	149	62	4	4	4	11	3	1	15	8	15	2	1	3	3	2	11

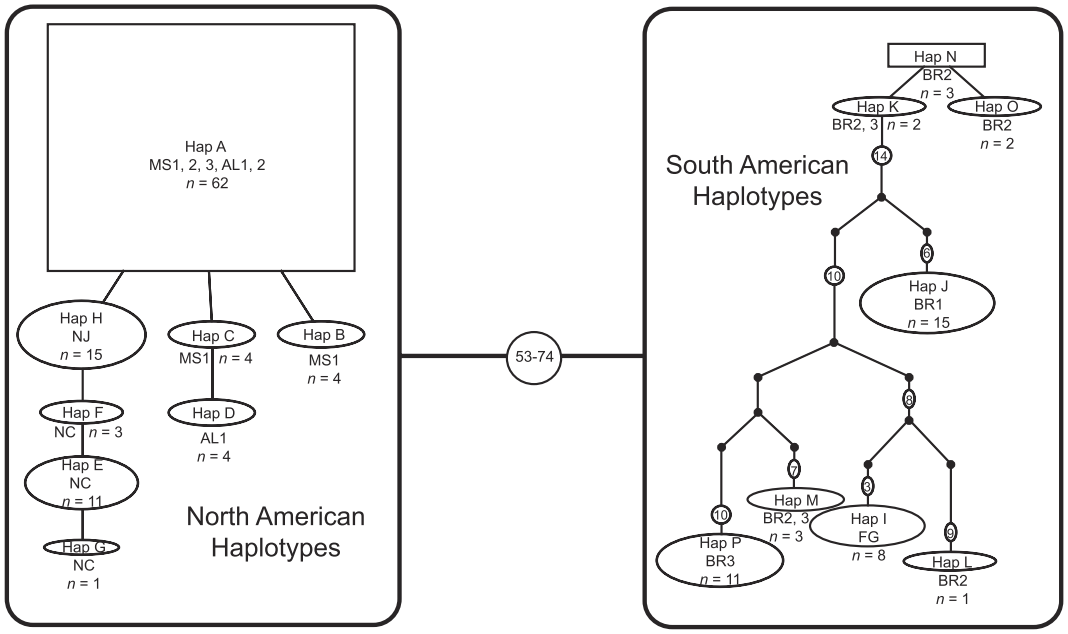

FIG. 2. Minimum spanning networks of *Batrachospermum macrosporum cox*2-3 spacer haplotypes from North and South America. *n* = sample numbers. A single line indicates one mutational step, filled circles represent missing intermediates, and circles with numbers specify multiple intermediate steps. The rectangle around haplotypes A and N signifies these as the most probable outgroups calculated using the TCS software. Haplotype and location designations as given in Table S1 (see the supplementary material).

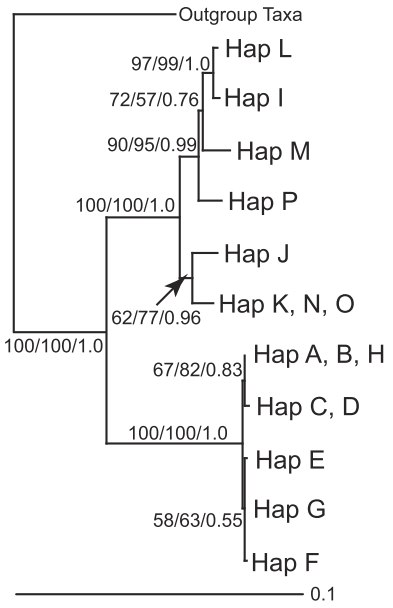

FIG. 3. Tree from ML (–Ln 7801.18755) analysis of *rbc*L gene sequence data showing the relationship among *Batrachospermum macrosporum* haplotypes. Haplotype designations as given in Table S1 (see the supplementary material). Support values for all analyses are shown as follows: MP bootstrap/ME bootstrap/Bayesian posterior probabilities.

locations MS2, MS3, NJ, AL1, AL2, and FG; dioecious thalli in NC and BR3; potentially dioecious thalli in MS1, BR1, and BR2 since no spermatangia were seen on the female thalli, but no male thalli were observed to confirm dioecy. No clear association of sexuality with haplotypes was evident.

DISCUSSION

The length (334–348 bp) of *cox*2-3 spacer region in *B. macrosporum* was within the range reported for select orders of marine red algae (Zuccarello et al. 1999). This length is somewhat shorter than that reported for *B. helminthosum* (370–371 bp), *B. arcuatum* (374–395 bp), and *Sirodotia delicatula* (376 bp) (Chiasson et al. 2003, 2005, Necchi and Vis 2005). The variation (<0.9%) among the North American *B. macrosporum* haplotypes (A–H) sampled was lower than that (≤6.5%) reported for *B. helminthosum* in North America. However, *B. macrosporum* was from a single biome (coastal plain) with a somewhat restricted distribution, whereas *B. helminthosum* was from throughout eastern North America. The variation (≤13.1%) among haplotypes (I–O) from South America was greater than that reported in *B. helminthosum*, but the South American locations were spread over a greater geographic distance. The variation between the two continents is greater than that seen for *B. helminthosum* from a single continent, but similar to that (≤13.1%) found among *Spyridia filamentosa* individuals from distant locations in the Pacific Ocean (Zuccarello et al. 2002).

The similarity in the haplotypes from North American locations, all within the coastal plain, may have been the result of colonization of suitable habitat from a refugium or the lack of in situ genetic

divergence over a long period of time. The former possibility is more likely, given the geologic history of these coastal areas and similar zoogeographic patterns observed in some fish species (Riggs 1984, Hocutt et al. 1986). In South America, it would appear that the Amazonian region is a center of diversity, perhaps having been a refugium during the last glaciation and a source of populations to colonize other regions of South and North America (Lynch 1988). Bermingham and Martin (1998) analyzed mitochondrial gene trees of neotropical freshwater fishes and produced evidence showing two to three waves of invasion (from late Miocene to Pleistocene) from South to North America through the Isthmian Central America as a linking corridor from putative source populations in northwestern Colombia. It is reasonable to infer that similar colonization events have occurred in freshwater algae, such as *B. macrosporum*.

Of the 11 locations sampled, eight revealed only one or two *cox*2-3 spacer haplotypes among the individuals. This result was congruent with the phylogeographic study of *B. helminthosum*, which showed only one location with more than two haplotypes (Chiasson et al. 2003). That study presented one location with three closely related and one divergent haplotype (6.5%), encompassing a large proportion of the genetic variation revealed from across eastern North America. Likewise, in the present study, the two locations in Brazilian Amazonia (BR2 and BR3) had five and three haplotypes, respectively, that diverged as much as 13%. The previous researchers suggested two possibilities for such genetic diversity within a given location: multiple colonization events or the population being more ancient, with the site being a glacial refugium (Chiasson et al. 2003). Again, for this study, each of these scenarios could account for the diversity shown since the Amazonian region is both an overwintering ground for numerous birds and a Pleistocene glacial refugium (DeGraaf and Rappole 1995, Brown and Lomolino 1998).

The phylogeographic data for *B. macrosporum* show a distinct dichotomy between specimens collected in locations from North and South America (Figs. 2 and 3). This result is somewhat unexpected given the numerous flyways between eastern North America and South America and the potential for birds to be dispersal vectors of freshwater algae (Proctor 1966, Atkinson 1972, DeGraff and Rappole 1995). These data would suggest that the populations on these two continents diverged long ago and remain isolated. This observation is supported by the fact that no desiccation-resistant stage is known for freshwater reds in the Batrachospermales (Sheath and Hambrook 1990), and that narrow endemics are not uncommon worldwide (Kumano 2002). Although some taxa (i.e., *Sirodotia suecica* and *B. atrum*) from different continents have identical *rbc*L gene sequences (Vis and Entwisle 2000), there has been limited sampling and no corroborating evidence in favor of long-distance dispersal over vicariance origins followed by minimal genetic change.

The amount of genetic variation observed in the *cox*2-3 spacer and *rbc*L gene data for *B. macrosporum* could indicate cryptic species or incipient speciation rather than phylogenetic signal of a single taxon. This hypothesis cannot be ruled out by the sequence data. Nevertheless, two of the researchers reexamined the specimens in terms of morphological variation to support this split. None of the currently recognized taxonomic characters could be partitioned based on this dichotomy, nor were any new features observed that would warrant describing a new taxon. It was noted that some locations had thalli that were clearly monoecious and others that were dioecious, but both types of breeding systems were seen on both continents (Necchi 1990, Sheath et al. 1994). There is little information on breeding system for this taxon in the literature. Both Necchi (1990) and Kumano (2002) state that *B. macrosporum* is dioecious and rarely polyecious, but this tendency may be due to the fact that most samples from Brazil are dioecious. Sheath et al. (1994) did not include this qualitative trait in their morphometric study of Section *Aristata*. Since breeding system did not support the genetic split, nor has it been used in the past to differentiate taxa in this section, we recommend that the circumscription of *B. macrosporum* be expanded to include both monoecy and dioecy. It is not uncommon for batrachospermalean taxa to have populations with either breeding system, as pointed out by Entwisle et al. (2004) in their discussion of monoecy/dioecy as a taxonomic character.

Based on the studies of *Batrachospermum* species to date, it would appear that there is considerable intraspecific genetic differentiation in the *cox*2-3 spacer region of individuals among locales, but little variation within a location, as previously discussed for *B. helminthosum* and *B. macrosporum*. Likewise, a study of *B. arcuatum* individuals among streams from Hawaii, Kauai, and Oahu revealed a single haplotype per stream segment, with 2.3%–2.9% genetic variation among the streams (Chiasson et al. 2007). Necchi and Vis (2005) showed a single haplotype for *S. delicatula* present in three geographically close streams. It would appear that colonization of a site might be rare, with a single event and subsequent proliferation playing a major role in the presence of *Batrachospermum* in a stream segment. It cannot be determined from the present data if two or more haplotypes at a single location are the result of colonization or in situ genetic differentiation. However, in most cases, the haplotypes only differ by one or two nucleotide changes, and it seems more likely that it is a result of sexual reproduction in stream, which is quite frequent in *Batrachospermum*. Interestingly, the two sites from Brazilian Amazonia have haplotypes that differ greatly, perhaps resulting

from multiple colonization events. As more studies are conducted on the phylogeography in freshwater red algae, the role of dispersal and vicariance for these organisms should become clearer.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Location and GenBank accession information for *Batrachospermum macrosporum* haplotypes from streams in North and South America.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00540.x

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