JSE Journal of Systematics and Evolution

doi: 10.1111/jse.12502

Research Article

Maintenance of genetic and morphological identity in two sibling *Syrrhopodon* species (Calymperaceae, Bryopsida) despite extensive introgression

Marta R. Pereira[1†], Alice Ledent[2†], Patrick Mardulyn[3], Charles E. Zartman[1†], and Alain Vanderpoorten[2†*]

[1]Department of Biodiversity, National Institute for Amazonian Research, Petrópolis, CEP Manaus Amazonas 69060-001, Brazil
[2]Institute of Botany, University of Liège, B22 Sart Tilman Liège 4000, Belgium
[3]Evolution Biologique et Ecologie, Université Libre de Bruxelles, Brussels 1050, Belgium
[†]These authors contributed equally to the work.
*Author for correspondence. E-mail: a.vanderpoorten@uliege.be
Received 28 February 2019; Accepted 16 April 2019; Article first published online 29 April 2019

Abstract Bryophytes are a group of land plants in which the role of hybridization has long been challenged. Using genotyping by sequencing to circumvent the lack of molecular variation at selected loci previously used for phylogeny and morphology, we determine the level of genetic and morphological divergence and reproductive isolation between the sibling *Syrrhopodon annotinus* and *S. simmondsii* (Calymperaceae, Bryopsida) that occur in sympatry but in different habitats in lowland Amazonian rainforests. A clear morphological differentiation and a low (0.06), but significant *F*st derived from the analysis of 183 single nucleotide polymorphisms were observed between the two species. Conspecific pairs of individuals consistently exhibited higher average kinship coefficients along a gradient of geographic isolation than interspecific pairs. The weak, but significant genetic divergence observed is consistent with growing evidence that ecological specialization can lead to genetic differentiation among bryophyte species. Nevertheless, the spatial genetic structures of the two species were significantly correlated, as evidenced by the significant slope of the Mantel test based on kinship coefficients between pairs of interspecific individuals and the geographic distance separating them. Interspecific pairs of individuals are thus more closely related when they are geographically closer, suggesting that isolation-by-distance is stronger than the interspecific reproductive barrier and pointing to interspecific gene flow. We conclude that interspecific introgression, whose role has long been questioned in bryophytes, may take place even in species wherein sporophyte production is scarce due to dioicy, raising the question as to what mechanisms maintain differentiation despite weak reproductive isolation.
Key words: introgression, isolation-by-distance, mosses, reproductive isolation, spatial genetic structure.

1 Introduction

Interspecific hybridization has long been recognized as a widespread and important phenomenon in plant evolution (Payseur & Rieseberg, 2016; Alix et al., 2017). Alloploidization, which involves whole genome duplication with the chromosome numbers of the two parents being summed in the hybrid species, has long been perceived as the dominant form of hybrid speciation (Abbott et al., 2010). Homoploid speciation, the evolution of a species without change in chromosome number, is thought to be a rarer form of speciation, although its detectability might increase with the advances of genomics (Abbott et al., 2010).

In fact, genomic methods have dramatically improved our ability to detect introgression and have expanded the number of taxa amenable to a detailed study of hybridization (Goulet et al., 2017). By far the most widely-used approach to detect hybridization with molecular data is the use of model-based methods to infer global (genome-average) and local (locus-specific) ancestry from population variation data, such as STRUCTURE (Porras-Hurtado et al., 2013). These methods rely, however, on a series of assumptions, including no or weakly linked loci, which are not met in clonal organisms. Alternatively, Hardy & Vekemans (2001) introduced a method to evidence interspecific gene flow by contrasting the effect of reproductive barriers between species and isolation by distance within species on population genetic structure. Although this method does not make any assumptions on the genetic structure, an important requirement is that samples of each species show similar geographic distributions to ensure that, for all types of pairwise comparisons (i.e., within and between species), a full range of geographic distances between samples is present. Since this method integrates geographic distance with genetic relatedness, it allows for determining the spatial scale at which interspecific gene flow occurs.

Here, we apply this approach to address the question of hybridization in bryophytes, wherein, despite substantial evidence for allopolyploidization (e.g., Barbulescu et al., 2017; Karlin & Smouse, 2017; Nieto-Lugilde et al., 2018) and associated shifts in sexual systems (Perley & Jesson, 2015), hybridization has long been perceived as nothing more than "an ephemeral and evolutionarily insignificant phenomenon" (Natcheva & Cronberg, 2004). The reason for this, perhaps, is the fact that bryophytes are typically seen as mostly clonal organisms. Two-third of the moss species are dioicous and rarely produce sporophytes. Monoicous species, in turn, are characterized by high rates of selfing (Eppley et al., 2007; Hutsemékers et al., 2013). Such features do not, in principle, promote hybridization. Actual evidence for recombination of nuclear markers associated with sympatric occurrence of phenotypically intermediate specimens is indeed extremely limited (Shaw, 1998; Natcheva & Cronberg, 2007), and hybridization in bryophytes has been inferred from equivocal evidence such as intermediate morphology (see Natcheva & Cronberg, 2004 for review) and incongruence between chloroplast and nuclear DNA sequences (e.g., Hedenäs, 2015, 2017).

In the present paper, we focus on the species pair formed by *Syrrhopodon simmondsii* and *S. annotinus* (Calymperaceae), which appears as an ideal model to test for reproductive isolation in mosses. These two species are sympatric in their Amazonian range where they occupy different micro-habitats, *S. annotinus* mostly occurring on mineral substrates such as rock and soil, whereas *S. simmondsii* tends to occur on organic substrates such as dead wood, humus, and trees (Reese, 1993), avoiding the common problem that ecological differentiation is confounded with distance and/or barriers to dispersal (Weber et al., 2017). Specimens of *S. annotinus* and *S. simmondsii* clustered within a clade in phylogenetic analyses based on a combination of five chloroplast and one nuclear region (Pereira et al., 2019), but the lack of molecular variation within that clade called for the use of more variable markers. Single nucleotide polymorphisms (SNPs) have been successfully employed in fine-scale population genetics of mosses (Rosengren et al., 2015, 2016) and we, therefore, built an SNP library from a modified genotyping by sequencing (GBS) protocol to test the morphological species concept of *S. annotinus* and *S. simmondsii,* address the following questions and test the following hypotheses: to what extent are the spatial genetic and morphological structures of the two species correlated? If *S. annotinus* and *S. simmondsii* are reproductively isolated, we expect that reproductive barriers are stronger than isolation-by-distance effects, and hence, that there is no relationship between the genetic and morphological similarity of pairs of interspecific individuals and the geographic distance separating them (H1). If the two species are conspecific, we expect that the regression curves between conspecific and interspecific pairs of individuals and geographic distance are overlapping (H2). If interspecific gene flow occurs between two genetically diverging species, we expect that the genetic and morphological similarity of pairs of individuals is higher in conspecific than in interspecific comparisons, but that the regression slope between interspecific genetic, and potentially also morphological, similarity and geographic distance is significant (H3).

2 Material and Methods

2.1 Sampling and molecular protocols

Forty-eight specimens, including 25 of *Syrrhopodon annotinus* and 23 of *S. simmondsii*, were sampled from a 42 640 km^2 area of lowland (<100 m) rainforest in the Rio Negro Basin North of Manaus (Table S1; Data S1). Climatic conditions, characterized by yearly average temperatures of 27.5 °C and 2145 mm of rainfall, are homogeneous across the study area. Main forest types include dense rainforests, more open forest types developing on white sand, and seasonally inundated forests. *Syrrhopodon annotinus* and *S. simmondsii* were only found in the white-sand forest (Sierra et al., 2018). The study area lays in the core distribution area of these two species, *S. annotinus* being an Amazonian endemic while *S. simmondsii* is endemic to northern South America (Reese, 1993). The sampling was organized to include a range of geographic distance among specimens from 0 to 250 km, in local sympatry (specimens of the two species at a distance of less than 25 m) or in allopatry (only specimens from one species found within the nearest 25 m). Specimens were collected in tubes and readily dried-out in silica gel.

Samples were frozen in liquid nitrogen for 5 min and DNA extracted using the DNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands). Single nucleotide polymorphism (SNP) libraries were prepared based on a GBS protocol (Elshire et al., 2011) modified as follows: (i) 100 ng DNA were digested with ApeKI and (ii) a double size selection of DNA fragments of 150–400 bp was performed using SPRI beads to only target fragments of sequenceable size. Fragments were amplified with a Q5 Hot Start High-Fidelity DNA Polymerase NEB (New England Biolabs, Evry, France) to enhance specificity and reduce amplification errors. A scalable complexity reduction was achieved by using longer 3′ primers that cover the entire common adapter, the 3′ restriction site and extend 1 or 2 bases into the insert following Sonah et al. (2013). PCR products were purified using AMPure XP beads. We gave each individual a forward and a reverse 4–8 bp long barcode (one at the 5′ end and one at the 3′ end), such that each individual had a unique barcode and could be multiplexed with all other individuals. These barcodes were selected from the 384 barcodes specifically designed to be used with ApeKI (http://www.maizegenetics.net/genotyping-by-sequencing-gbs). The concentration of PCR products was assessed by fluorometry with the Quant-iT PicoGreen dsDNA Assay Kit before multiplexing to ensure the equimolarity of PCR products in final libraries. Distribution of fragment sizes for each library was analyzed by capillary electrophoresis with a QIAxcel to look for any remaining adaptor (around 128 bp). If present, adaptors were removed by selecting fragments of >150 bp on a polyacrylamide gel. Paired-end sequencing (2 × 75 bp) of the libraries was performed with an Illumina NextSeq. 500 sequencer in low-output mode (i.e., 130 000 000 reads per line).

Sequences of the adaptors at both 3′ and 5′ ends of each read as well as low-quality sequences (Phred score <20) at both ends were removed with cutadapt 1.16 (https://cutadapt.readthedocs.io). ipyrad 0.7.28 (https://ipyrad.readthedocs.io) was then used to demultiplex the libraries and to cluster alleles that diverged by less than 15% within and then among individuals. Following Paris et al. (2017), we

set the minimum number of raw reads required to form an allele to 3. Due to the haploid condition of the target species, loci with more than one allele per individual were discarded. We then discarded any locus that was sequenced in less than 30% of the individuals.

2.2 Statistical analyses

To test the morphological species concept of *S. annotinus* and *S. simmondsii,* we performed analyses at the level of allelic combinations on the one hand, and allele frequencies on the other. We therefore performed a principal component analysis of the SNP data matrix to identify combinations of alleles that best discriminate specimens and then employed a one-way analysis of variance to determine whether there were significant differences of the average score of specimens morphologically assigned to either species, and hence, determine whether the observed genetic segregation corresponds to the distinction between the two species. We further computed the *F*st between groups of specimens morphologically assigned to either *S. annotinus* or *S. simmondsii* to seek for genetic differences at the level of allele frequencies. The significance of *F*st was assessed by 1000 random permutations of individuals among species. To test the hypothesis that the two species are reproductively isolated, we computed Mantel tests between kinship coefficients *F*ij (Loiselle et al., 1995) (i) for pairs of conspecific individuals and (ii) for pairs of interspecific individuals, and the geographic distance separating them. The significance of the slope associated with the Mantel test among conspecific pairs was assessed by random permutations of 1000 individuals among localities. For comparisons between species, care must be taken in the interpretation of significance tests when spatial autocorrelation occurs within each species (Hardy & Vekemans, 2001). This problem arises because randomization of spatial positions not only uncouples the spatial structures of both species, as needed, but also suppresses the spatial autocorrelation within each species. Thus, for comparisons between species, the randomization tests may overestimate the association between geographic distances and genetic distances between interspecific individuals. Therefore, to assess the significance of the slope of the Mantel tests among non-conspecific species pairs, we implemented a Jacknife test, wherein the slope was recalculated after successively pruning one locus from the data at a time, to estimate the standard deviation of the slope across loci, and hence, determine whether its 95% confidence interval encompasses 0. One-way analysis of variance was employed to test the hypothesis that average kinship coefficients between pairs of conspecific individuals were higher than between interspecific pairs of specimens. All computations were performed with Spagedi 1.5 (Hardy & Vekemans, 2002).

To compare the fine-scale genetic structure of *S. annotinus* and *S. simmondsii* with that of angiosperms, we also computed the *Sp* statistics, which characterizes the rate of decrease of pairwise kinship coefficients between individuals with the logarithm of the distance (Vekemans & Hardy, 2004). The *Sp* statistics varies as a function of the mating system and dispersal traits, low values typically characterizing organisms with high dispersal capacities and outbreeding mating systems. The *Sp* statistics is measured as $-\hat{b}_F/(1-\hat{F}_1)$, where $-\hat{b}_F$ is the regression slope on the logarithm of distance and F_1 is the mean kinship coefficient between individuals belonging to the first distance interval that includes all pairs of neighbors, and is computed for comparison with the values reported in angiosperms (Vekemans & Hardy, 2004).

2.3 Morphological analyses

Fourteen variable gametophytic characters (Table 1) were scored on the specimens used for molecular analysis. Ten randomly selected leaves were sampled from each of three randomly selected shoots per collection. To determine whether there is a continuous morphological range of variation between the two species, a principal component analysis was performed, and significant differences in the average score of specimens assigned to each species, respectively, on the first two axes, were sought using one-way analysis of variance. To test the hypothesis that morphological differentiation is consistently higher in interspecific than in intraspecific comparisons along a gradient of geographic distance (H2), we computed Euclidian distances of both pairs of conspecific and interspecific individuals separately. We then computed the correlation coefficient between Euclidian distances for both intra- and interspecific comparisons and geographic distance, and employed one-way analysis of variance to test that average morphological differences were significantly higher in inter- than intraspecific comparisons.

3 Results

An average total of 1.5 million reads per individual were obtained. From those 1.5 million reads per individual, the clustering of alleles diverging by a maximum of 15% within individuals led to an average of 750 000 allele clusters per individual. The minimum number of raw reads required to form an allele (set to 3) led to the loss of an average of 86% of the allele clusters. The filtering-out of heterozygous allele clusters led to the loss of another 2% of them. From the resulting matrix of 14 000 loci on average per specimen, we ended-up, after clustering loci diverging by a maximum of 15% among individuals, filtering-out loci and individuals with >75 and 90% of missing data, respectively, with a matrix including 40 specimens listed in Table 1 from an initial number of 48 and 183 loci. In that matrix (Data S1), $37.2 \pm 16.1\%$ of the loci were sequenced on average per specimen.

The analyses seeking for significant differences between *S. annotinus* and *S. simmondsii* at the level of allele combinations on the one hand, and allele frequencies on the other, revealed an extremely weak genetic divergence between the two species. Thus a weak and marginally significant ($F = 4.1$, $P = 0.05$) average difference was found between the scores of individuals morphologically assigned to either *S. annotinus* or *S. simmondsii* along the first axis of the principal component analysis of the SNP datamatrix (Fig. 1). The *F*st between specimens morphologically assigned to either *S. annotinus* or *S. simmondsii* was 0.059 ($P = 0.004$), reflecting the weak, but significant difference of allele frequencies between the two species.

Average kinship coefficients along a geographic gradient were consistently higher for conspecific comparisons than

Table 1 Morphological characters scored to describe the differentiation between *Syrrhopodon annotinus* and *S. simmondsii* and among conspecific specimens

Characters	Description
Leaf characters	
1. Leaf length (LL)	0 = short (<2.5 mm); 1 = long (2.5–5 mm); 2 = very long (>5 mm)
2. Leaf width (LW)	0 = thin (<0.5 mm); 1 = wide (>0.5 mm)
3. Leaf serration (LS)	0 = absent; 1 = < 10 teeth present on either leaf side; 2 = with more than 10 noticeable teeth on each side of leaf
4. Costa width (CW)	0 = thin (10–60 μm); 1 = medium (60–200 μm); 2 = thick (>200 μm)
5. Gemmiferous leaves (GL)	0 = absent; 1 = present
Leaf margin	
6. Hyaline border (HB)	0 = marginal; 1 = submarginal
Cancellinae	
7. Cancellinae cell length (CL)	0 = short (15–50 μm); 1 = long (>50 μm)
8. Cancellinae cell width (CCW)	0 = thin (7.5–12.5 μm); 1 = medium (20–30 μm); 2 = wide (>50 μm)
Chlorophyllose cells	
9. Chlorophyllose cell length (ChL)	0 = short (7–9 μm); 1 = long (9–12 μm)
10. Chlorophyllose cell width (ChW)	0 = thin; 1 = wide
11. Chlorocyst papillae (CP)	0 = absent; 1 = present
Stem characters	
12. Central strand (CS)	0 = absent; 1 = present
13. Stem thickness (ST)	0 = small (1–5 mm); 1 = large (5–10 mm)
Plant characters	
14. Plant size (PS)	0 = small (<1 cm); medium sized (=1–2 cm); robust (≥2 cm)

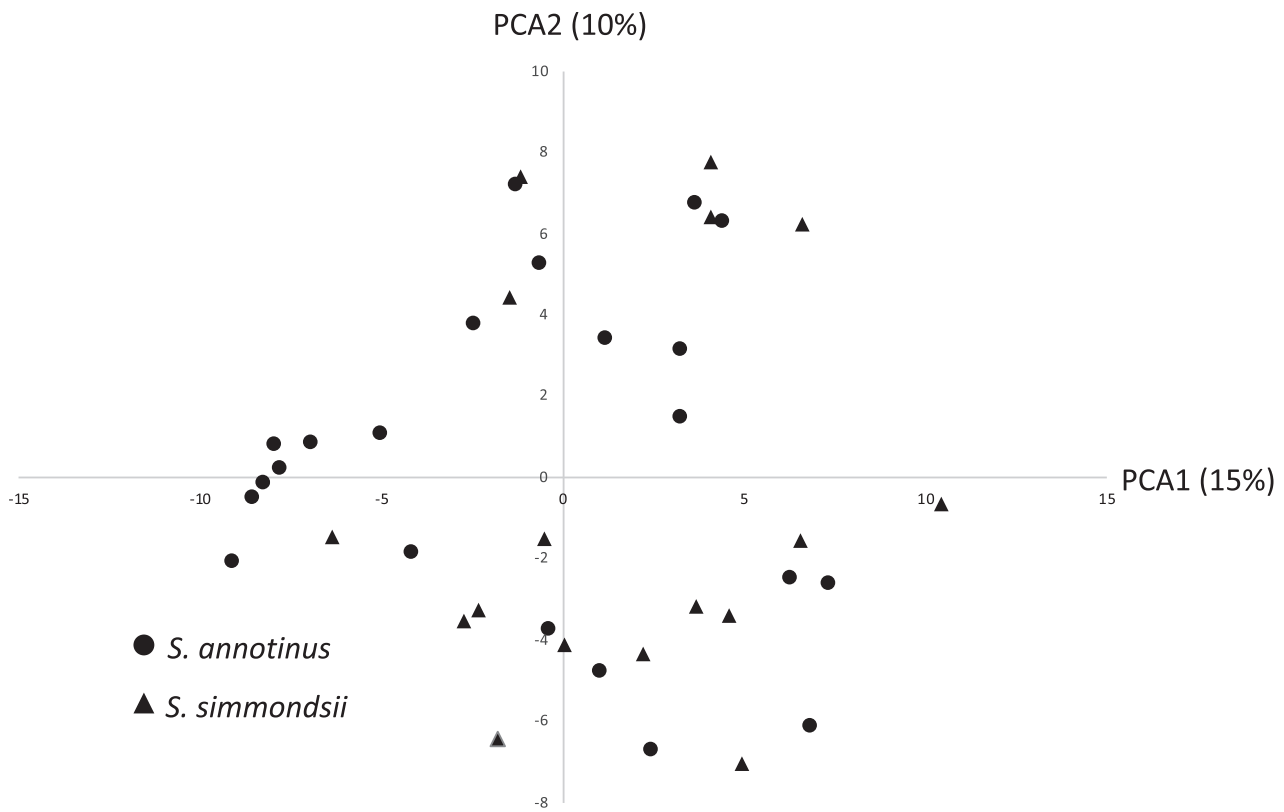

Fig. 1. Principal component analysis of genetic variation at 183 SNPs in the sibling moss species *Syrrhopodon annotinus* and *S. simmondsii.* Values in parentheses indicate the proportion of explained variance of the first two axes. SNPs, single nucleotid polymorphisms.

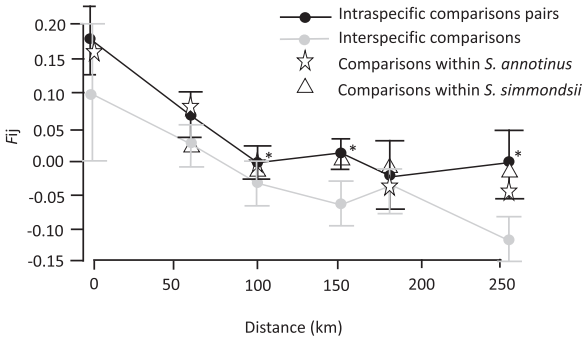

Fig. 2. Spatial autocorrelogram showing average kinship coefficients Fij (±SD) derived from the analysis of 183 SNPs per distance class between pairs of conspecific (black line) and interspecific (gray line) individuals of *Syrrhopodon annotinus* and *S. simmondsii* as a function of the distance between pairs of individuals. Stars indicate the confidence level (*<0.05) of the anova of intra- and interspecific comparisons at each distance class. SNPs, single nucleotid polymorphisms.

for interspecific comparisons, and significantly so in three out of the six comparisons (Fig. 2). The *Sp* statistics derived from the Mantel test based upon comparisons of conspecific pairs of individuals was 0.04 (0.043 for *S. annotinus* and 0.024 for *S. simmondsii*). Nevertheless, the spatial genetic structures of the two species were significantly correlated. In fact, the interval of confidence of the regression slopes between pairs of conspecific individuals (*b*-log slope = −0.033 ± 0.011; $P < 0.001$), and also between pairs of interspecific individuals (*b*-log slope = −0.034 ± 0.010), did not encompass 0, evidencing a significant pattern of isolation-by-distance in both cases.

The distribution of specimens along the first axes of the PCA of morphological characters, which accounted for 55 and 16% of the total variance, respectively, is shown in Fig. 3A. There were significant morphological differences between *S. annotinus* and *S. simmondsii*, as revealed by significant ANOVAS of the average score of specimens assigned to each species along PCA1 ($P < 0.001$), but not PCA2 ($P = 0.26$). PCA1 was mostly correlated negatively with chlorophyllose cell width, leaf serration, and presence/absence of chlorocyst papillae, and positively with plant size, leaf length, costa width, and stem thickness (Fig. 3B). None of the intra- and interspecific Euclidian distances were correlated with geographic distance (Fig. 4). Differences between pairs of specimens of different species were consistently significantly higher than between pairs of conspecific individuals across the geographic range (Fig. 4).

4 Discussion

The significant Fst between sympatric specimens assigned to *Syrrhopodon annotinus* and *S. simmondsii* indicates that reproductive isolation led to differences in allele frequencies between two sympatric species characterized by different habitat requirements. Shaw et al. (1987) similarly found significant differences in a sympatric pair of moss species (*Climacium americanum* and *C. dendroides*) from different habitats. Although it is not possible to determine whether habitat differentiation triggered or followed speciation, these observations are consistent with growing evidence that ecological specialization can lead to genetic differentiation within bryophyte species. These observations contrast with the hypothesis that physiological plasticity prevails over genetic specialization in bryophytes based on experimental work suggesting that, in contrast with the vast majority of angiosperm species, bryophyte species do not tend to develop ecotypes, but rather display an inherent broad ability to cope with environmental variation (Shaw, 1992). The results presented here contribute to growing evidence for genetic divergence (Szövényi et al., 2009; Hutsemékers et al., 2010; Pisa et al., 2013; Mikulaskova et al., 2015; Magdy

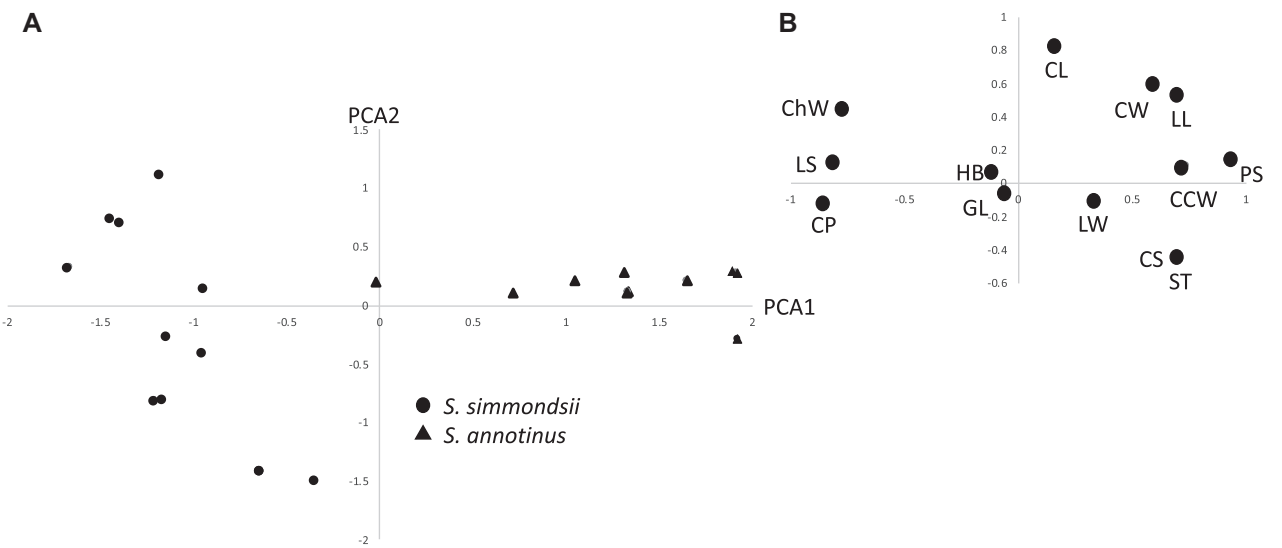

Fig. 3. Principal components analysis of 14 gametophytic characters in *Syrrhopodon annotinus* and *S. simmondsii*. **A**, Projection of the individuals onto the first two axes. **B**, Correlation between the variables (see Table 1 for abbreviations) and the axes.

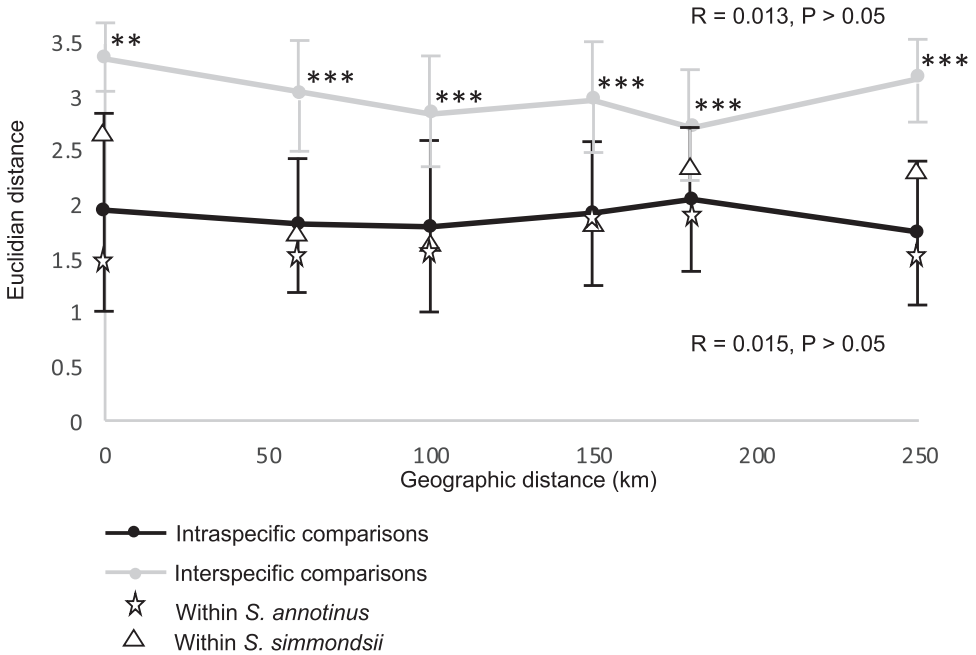

Fig. 4. Average ± SD of the Euclidian distance of morphological characters (Table 1) between pairs of conspecific (black line) and interspecific (gray line) individuals of *Syrrhopodon annotinus* and *S. simmondsii* as a function of the distance between pairs of individuals. Stars indicate the confidence level (**$P < 0.01$; ***$P < 0.001$) of the anova of intra- and interspecific comparisons at each distance class.

et al., 2016) and speciation (Johnson et al., 2015) along environmental gradients in bryophytes, suggesting that adaptation could play a more important role in shaping genetic patterns than previously thought. In particular, an increased role for ecological speciation contrasts with the hypothesis that the failure of bryophytes to radiate is caused by the limited importance of isolation-by-environment in the group (Patiño et al., 2014).

The slight, but significant, genetic divergence between *S. annotinus* and *S. simmondsii* (6% of the total variance of allele frequencies) and the significantly higher kinship coefficients in intraspecific than in interspecific pairs of individuals suggest that the sharp morphological differences between the two species cannot be simply explained by plasticity. Furthermore, no correlation between morphological variation and geographic distance was observed in *S. annotinus* and *S. simmondsii*. Interspecific morphological differences were consistently significantly higher than intraspecific differences across the geographic range investigated. This indicates that, in contrast to *S. leprieurii* (Pereira et al., 2013), the observed morphological differentiation between *S. annotinus* and *S. simmondsii* cannot be explained by an alternative hypothesis of geographic variation within a single species (Zapata & Jiménez, 2012).

However, despite the fact that pairs of conspecific individuals consistently exhibited higher average kinship coefficients than pairs of interspecific individuals, the slope of the Mantel test based on kinship coefficients between interspecific pairs was significantly spatially correlated. This indicates that isolation-by-distance is stronger than the interspecific reproductive barrier. Interspecific pairs of individuals are therefore more closely related when they are geographically closer, evidencing interspecific gene flow and strongly supporting Natcheva & Cronberg's (2004) suggestion that hybridization may be a common, yet largely overlooked, mechanism in bryophytes.

The strong isolation-by-distance pattern revealed here is consistent with the dispersal traits of the studied species, which are characterized by immersed sporophytes within perichaetial leaves, fairly large spores of >30 µm, the absence of a peristome, and infrequent gemmae and sporophyte production (Reese, 1993), together with extrinsic features of their environment of dense rainforests that is not prone to long-distance wind-dispersal. The *Sp* statistics of 0.04 lay in the upper limit of the range reported for species with gravity-dispersed seeds (0.028 ± 0.016) and is higher than the range reported for species characterized by wind (0.012 ± 0.012) and animal (0.008 ± 0.005)-dispersed seeds (Vekemans & Hardy, 2004). Such a poor dispersal capacity does not point to the transportation of diaspores by animals, which has been increasingly documented in bryophyte species from dense forest environments with low wind connectivity (Heinken et al., 2001; Parsons et al., 2007; Rudolphi, 2009; Wilkinson et al., 2017). The Sp statistics was, in turn, lower than that reported for plant species characterized by selfing (and even more, clonal) mating systems (0.14 ± 0.08), which is consistent with the hypothesis that interspecific gene flow may occur in sympatry. Individuals of *S. annotinus* and *S. simmondsii* in fact grow in the close vicinity of each other, so that sperm cells may reach the archegonia of a non-conspecific female individual. In a one-year monitoring survey of sex expression, Pereira et al. (2016) failed, however, to find expressed males and sporophytes in *S. annotinus*, while expressed males and

sporophytes were found in <1% and 5% of investigated individuals, respectively, in *S. simmondsii*. Since gametangia production was positively correlated with precipitation (Pereira et al., 2016), we hypothesize that interspecific gene flow reported here dates back to historical periods of increased sexual reproduction associated with historically wetter climates.

Our results point to three main conclusions. First, based on the fact that there is (i) a weak, but significant *F*st between sympatric specimens assigned to *S. annotinus* on the one hand, and *S. simmondsii* on the other, and that kinship coefficients are significantly lower in interspecific than in intraspecific comparisons and (ii) a sharp morphological differentiation, we tentatively suggest that *S. annotinus* and *S. simmondsii* represent two distinct species. However, the weakness of the reproductive barrier between them, evidenced by the significance of the regression slope of *F*ij in interspecific comparisons, suggests that *S. annotinus* and *S. simmondsii* diverged recently. During the speciation process, various aspects of lineage divergence, in fact, arise (De Queiroz, 2007). Sister species progressively diverge from each other over time, but the acquisition of the different properties defining them (when they become phenotypically diagnosable, reciprocally monophyletic for one or multiple loci, reproductively incompatible, ecologically distinct, etc.) is not simultaneous, potentially leading to conflicting assessments of species identity before and after the acquisition of any one of those properties.

Second, significant patterns of genetic structure were observed despite the comparatively low number of single nucleotide polymorphisms (SNPs) that could be expected with genotyping by sequencing (GBS) techniques and the fairly large amounts of missing data. By comparison with the results obtained in other plant studies using GBS or Rad-seq approaches, more than 1000 SNPs were obtained for each of the 663 individuals in *Protea repens* (Prunier et al., 2017), an average of 2778 independent SNP loci were obtained in 7 Australian Alpine species (Bell et al., 2018), and 17 982 SNPs were obtained in the fir *Keteleeria davidiana* with 50% missing data (Shih et al., 2018). The 183 SNPs obtained here are more comparable to the 63 SNPs obtained in the only other moss species that had been investigated to date using GBS or Rad-seq techniques, *Tetraplodon fuegianus*, in its northern hemisphere range (Lewis et al., 2017). The lower number of SNPs obtained in mosses may reflect a truly lower genetic diversity caused, among others, by high rates of clonality, but may also call for protocol improvements in these non-model organisms. Nevertheless, the present results, together with those of Lewis et al. (2017), suggest that SNP data are a promising tool for shallow systematics (Fernández-Mazuecos et al., 2018; Ding et al., 2019) and fine-scale genetic structure (Attard et al., 2018) in bryophytes when traditional markers such as low-copy nuclear genes and chloroplast genes are not polymorphic.

Third, the maintenance of low, but significant levels of genetic divergence and a high morphological differentiation despite weak reproductive isolation (in fact, weaker than the strength of isolation-by-distance) is puzzling. The rarity of intermediate hybrid phenotypes was previously reported in mosses, wherein it was interpreted in terms of higher viability of spores that had inherited most of the genome from one or the other parent, or the low survival of the recombinant gametophytes (Cronberg, 1996; Cronberg & Natcheva, 2002). In the case of *S. annotinus* and *S. simmondsii*, we suggest that two mechanisms may contribute to the observed differentiation. First, the intensity of recombination may be limited by the resistance of large parts of the genome against heterospecific genes, maintaining the genetic distinctness of the species (Natcheva & Cronberg, 2007). Second, habitat specialization may play a key role through the counter-selection of hybrids, calling for the implementation of experimental transplantations and fitness measurements.

Acknowledgements

Many thanks are due to three referees for their comments on the manuscript. AL and AV acknowledge financial support from the Belgian Funds for Scientific Research (F.R.S.-FNRS). Computational resources were provided by the Consortium des Équipements de Calcul Intensif funded by F.R.S.-FNRS (Grant 2.5020.11). Fieldwork was funded by the Projecto Bilateral Brasil/Belgica processo 49518/2013-3 (CNPq), and by the Conselho Nacional de Desenvolvimento Cientıfico e Tecnologico (CNPq, Grant No. 441590/2016-0) and the Fundacao de Amparo a Pesquisa do Estado do Amazonas (FAPEAM, Grant No. 015/2016) for funding through the project PELD/MAUA (Ecologia, Monitoramento e Uso Sustenta vel de Areas Umidas Amazonicas). CEZ acknowledges financial support from MCT/CNPq N8017/2013: Cooperacao Internacional – Acordos bilaterais. Collecting permits in protected areas were delivered by the Instituto Chico Mendes de Conservação de Biodiversidade.

References

Abbott RJ, Hegarty MJ, Hiscock SJ, Brennan AC. 2010. Homoploid speciation in action. *Taxon* 59: 1375–1386.

Alix K, Gérard PR, Schwarzacher T, Heslop-Harrison JSP. 2017. Polyploidy and interspecific hybridization: Partners for adaptation, speciation and evolution in plants. *Annals of Botany* 120: 183–194.

Attard CRM, Beheregaray LB, Möller LM. 2018. Genotyping-by-sequencing for estimating relatedness in nonmodel organisms: Avoiding the trap of precise bias. *Molecular Ecology* 18: 381–390.

Barbulescu EVI, Patzak SDF, Feldberg K, Schäfer-Verwimp A, Rycroft DS, Renner MAM, Heinrichs J. 2017. Allopolyploid origin of the leafy liverwort Plagiochila britannica (Plagiochilaceae). *Botanical Journal of the Linnean Society* 183: 250–259.

Bell N, Griffin PC, Hoffmann AA, Miller AD. 2018. Spatial patterns of genetic diversity among Australian alpine flora communities revealed by comparative phylogenomics. *Journal of Biogeography* 45: 177–189.

Cronberg N. 1996. Clonal structure and fertility in a sympatric population of the peat mosses, *Sphagnum rubellum* and *S. capillifolium*. *Canadian Journal of Botany* 74: 1375–1385.

Cronberg N, Natcheva R. 2002. Hybridization between the peat mosses, *Sphagnum capillifolium* and *S. quinquefarium* (Sphagnaceae, Bryophyta) as inferred by morphological characters and isozyme markers. *Plant Systematics and Evolution* 234: 53–70.

De Queiroz K. 2007. Species concepts and species delimitation. *Systematic Biology* 56: 879–886.

Ding X, Xiao JH, Li L, Conran GJ, Li J. 2019. Congruent species delimitation of two controversial gold-thread nanmu tree species based on morphological and restriction site-associated DNA sequencing data. *Journal of Systematics and Evolution* 57: 234–246.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One* 6: e19379.

Eppley SM, Taylor PJ, Jesson LK. 2007. Self-fertilization in mosses: A comparison of heterozygote deficiency between species with combined versus separate sexes. *Heredity* 98: 38–44.

Fernández-Mazuecos M, Mellers G, Vigalondo B, Sáez L, Vargas P, Glover BJ. 2018. Resolving recent plant radiations: Power and robustness of Genotyping-by-Sequencing. *Systematic Biology* 67: 250–268.

Goulet EB, Roda F, Hopkins R. 2017. Hybridization in plants: Old ideas, new techniques. *Plant Physiology* 173: 65–78.

Hardy OJ, Vekemans X. 2001. Patterns of allozyme variation in diploid and tetraploid *Centaurea jacea* at different spatial scales. *Evolution* 55: 943–954.

Hardy OJ, Vekemans X. 2002. SPAGeDi: A versatile computer program to analyze spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618–620.

Hedenäs L. 2015. Molecular and morphological incongruence among the genera around *Sarmentypnum* (Bryophyta: Calliergonaceae). *Nova Hedwigia* 100: 279–292.

Hedenäs L. 2017. Three molecular markers suggest different relationships among three *Drepanocladus* species (Bryophyta: Amblystegiaceae). *Plant Systematics and Evolution* 303: 521–529.

Heinken T, Lees R, Raudnitschka D, Rung S. 2001. Epizoochorous dispersal of bryophyte stem fragments by roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*). *Journal of Bryology* 23: 293–300.

Hutsemékers V, Hardy O, Mardulyn P, Shaw A, Vanderpoorten A. 2010. Macroecological patterns of genetic structure and diversity in the aquatic moss *Platyhypnidium riparioides*. *New Phytologist* 185: 852–864.

Hutsemékers V, Hardy OJ, Vanderpoorten A. 2013. Does water facilitate gene flow in spore-producing plants? Insights from the fine-scale genetic structure of the aquatic moss *Rhynchostegium riparioides*. *Aquatic Botany* 108: 1–6.

Johnson MG, Granath G, Tahvanainen T, Pouliot R, Stenøien HK, Rochefort L, Rydin H, Shaw AJ. 2015. Evolution of niche preference in *Sphagnum* peat mosses. *Evolution* 69: 90–103.

Karlin EF, Smouse PE. 2017. Allo-allo-triploid *Sphagnum×falcatulum*: Single individuals contain most of the Holantarctic diversity for ancestrally indicative markers. *Annals of Botany* 120: 221–231.

Lewis LR, Biersma EM, Carey SB, Holsinger K, McDaniel SF, Rozzi R, Goffinet B. 2017. Resolving the northern hemisphere source region for the long-distance dispersal event that gave rise to the South American endemic dung moss *Tetraplodon fuegianus*. *American Journal of Botany* 104: 1651–1659.

Loiselle BA, Sork VL, Nason J, Graham C. 1995. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany* 82: 1420–1425.

Magdy M, Werner O, McDaniel SF, Goffinet B, Ros RM. 2016. Genomic scanning using AFLP to detect loci under selection in the moss *Funaria hygrometrica* along a climate gradient in the Sierra Nevada Mountains, Spain. *Plant Biology* 18: 280–288.

Mikulaskova E, Hajek M, Veleba A, Johnson MG, Hajek T, Shaw AJ. 2015. Local adaptations in bryophytes revisited: The genetic structure of the calcium-tolerant peatmoss *Sphagnum warnstorfii* along geographic and pH gradients. *Ecology & Evolution* 5: 229–242.

Natcheva R, Cronberg N. 2004. What do we know about hybridization among bryophytes in nature? *Canadian Journal of Botany* 82: 1687–1704.

Natcheva R, Cronberg N. 2007. Recombination and introgression of nuclear and chloroplast genomes between the peat mosses, *Sphagnum capillifolium* and *Sphagnum quinquefarium*. *Molecular Ecology* 16: 811–818.

Nieto-Lugilde M, Werner O, McDaniel SF, Koutecký P, Rizk SM, Ros RM. 2018. Peripatric speciation associated with genome expansion and female-biased sex ratios in the moss genus *Ceratodon*. *American Journal of Botany* 105: 1009–1020.

Paris JR, Stevens JR, Catchen JM. 2017. Lost in parameter space: A road map for STACKS. *Methods in Ecology and Evolution* 8: 1360–1373.

Parsons JG, Cairns A, Johnson CN, Robson SKA, Shilton LA, Westcott DA. 2007. Bryophyte dispersal by flying foxes: A novel discovery. *Oecologia* 152: 112–114.

Patiño J, Carine MA, Fernández-Palacios JM, Otto R, Schaefer H, Vanderpoorten A. 2014. The anagenetic world of spore-producing land plants. *New Phytologist* 201: 305–311.

Payseur BA, Rieseberg LH. 2016. A genomic perspective on hybridization and speciation. *Molecular Ecology* 25: 2337–2360.

Pereira MR, Dambros CS, Zartaman CE. 2013. Will the real *Syrrhopodon leprieurii* please stand up? The influence of topography and distance on phenotypic variation in a widespread Neotropical moss. *The Bryologist* 116: 58–64.

Pereira MR, Dambros CS, Zartman CE. 2016. Prezygotic resource-allocation dynamics and reproductive trade-offs in Calymperaceae (Bryophyta). *American Journal of Botany* 103: 1–9.

Pereira MR, Amorim BS, Sierra AM, McDaniel S, Payton A, Carey SB, Câmara PEAS, Zartman CE. 2019. Advances in Calymperaceae (Dicranidae, Bryophyta): Intra-familial phylogenetic relationships, geographical origin and divergence times. *The Bryologist*. In press.

Perley DS, Jesson LK. 2015. Hybridization is associated with changes in sexual system in the bryophyte genus *Atrichum*. *American Journal of Botany* 102: 555–565.

Pisa S, Werner O, Vanderpoorten A, Magdy M, Ros RM. 2013. Altitudinal genetic differentiation of the cosmopolitan moss *Bryum argenteum* in the mountains of Sierra Nevada (Spain, SW Europe). *American Journal of Botany* 100: 2000–2008.

Porras-Hurtado L, Ruiz Y, Santos C, Phillips C, Carracedo A, Lareu MV. 2013. An overview of STRUCTURE: Applications, parameter settings, and supporting software. *Frontiers in Genetics* 29: 98.

Prunier R, Akman M, Kremer CT, Aitken N, Chuah A, Borevitz J, Holsinger KE. 2017. Isolation by distance and isolation by environment contribute to population differentiation in *Protea repens* (Proteaceae L.), a widespread South African species. *American Journal of Botany* 104: 674–684.

Reese WD. 1993. Calymperaceae. *Flora Neotropica* 58: 1–101.

Rosengren F, Hansson B, Cronberg N. 2015. Population structure and genetic diversity in the nannandrous moss *Homalothecium lutescens*: Does the dwarf male system facilitate gene flow? . *BMC Evolutionary Biology* 15: 1–12.

Rosengren F, Cronberg N, Hansson B. 2016. Balance between inbreeding and outcrossing in a nannandrous species, the moss *Homalothecium lutescens*. *Heredity* 116: 107–113.

Rudolphi J. 2009. Ant-mediated dispersal of asexual moss propagules. *The Bryologist* 112: 73–79.

Shaw JT, Meagher TR, Harley P. 1987. Electrophoretic evidence of reproductive isolation between two varieties of the moss, *Climacium americanum*. *Heredity* 59: 337–343.

Shaw AJ. 1992. The evolutionary capacity of bryophytes and lichens. In: Bates JW, Farmer AM eds. *Bryophytes and lichens in a changing environment*. London: Clarendon Press. 362–380.

Shaw AJ. 1998. Genetic analysis of a hybrid zone in *Mielichhoferia* (Musci). In: Bates JW, Ashton NW, Duckett JG eds. *Bryology for the twenty-first century*. Leeds: Maney Publishing and the British Bryological Society. 161–174.

Shih KM, Chang CT, Chung JD, Chiang YC, Hwang SY. 2018. Adaptive genetic divergence despite significant isolation-by-distance in populations of Taiwan Cow-Tail Fir (*Keteleeria davidiana* var. *formosana*). *Frontiers in Plant Sciences* 9: 92.

Sierra AM, Vanderpoorten A, Gradstein SR, Pereira MR, Passos Bastos CJ, Zartman CE. 2018. Bryophytes of Jau´ National Park (Amazonas, Brazil): Estimating species detectability and richness in a lowland Amazonian megareserve. *The Bryologist* 121: 571–588.

Sonah H, Bastien M, Iquira E, Tardivel A, Légaré G, Boyle B, Normandeau E, Laroche J, Larose S, Jean M, Belzile F. 2013. An improved Genotyping by Sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. *PLoS One* 8: e54603.

Szövényi P, Hock Z, Korpelainen H, Shaw AJ. 2009. Spatial pattern of nucleotide polymorphism indicates molecular adaptation in the bryophyte *Sphagnum fimbriatum*. *Molecular Phylogenetics and Evolution* 53: 277–286.

Weber JN, Bradburd GS, Stuart YE, Stutz WE, Bolnick DL. 2017. Partitioning the effects of isolation by distance, environment, and physical barriers on genomic divergence between parapatric threespine stickleback. *Evolution* 71: 342–356.

Vekemans X, Hardy OJ. 2004. New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology* 13: 921–935.

Wilkinson DM, Lovas-Kiss A, Callaghan DA, Green AJ. 2017. Endozoochory of large bryophyte fragments by waterbirds. *Cryptogamie Bryologie* 38: 223–228.

Zapata F, Jiménez I. 2012. Species delimitation: Inferring gaps in morphology across geography. *Systematic Biology* 61: 179–194.

Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12502/suppinfo:

Table S1. Voucher information and GenBank accession numbers (GB) from BioProject PRJNA530510.

Data S1. SNP dataset for *Syrrhopodon annotinus* and *S. simmondsii* from Amazonas (Brazil) from GBS data, and Voucher information and GenBank accession numbers (GB) from BioProject PRJNA530510, available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.5tk616c