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Delimiting species boundaries within the Neotropical bamboo *Otatea* (Poaceae: Bambusoideae) using molecular, morphological and ecological data

Eduardo Ruiz-Sanchez *, Victoria Sosa

Departamento de Biologia Evolutiva, Instituto de Ecologia, A. C., Apartado Postal 63, 91000 Xalapa, Veracruz, Mexico

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ABSTRACT

Species delimitation is a task that has engaged taxonomists for more than two centuries. Recently, it has been demonstrated that molecular data and ecological niche modeling are useful in species delimitation. In this paper multiple data sets (molecular, morphological, ecological) were utilized to set limits for the species belonging to the Neotropical bamboo *Otatea*, because there is disagreement about species circumscriptions and also because the genus has an interesting distribution, with most of its populations in Mexico and a single disjunct population in Colombia. Molecular and morphological phylogenetic analyses recovered trees with conflicting topologies. Tree-based morphological and character-based analyses recognized the same entities. Ecological niche models and PCA/MANOVAS agreed with the recognition of the same entities that resulted from the morphological analyses. Morphological analyses retrieved clades supported by diagnostic characters and coherent geographical distributions. Based on these results seven entities should be recognized in *Otatea*, instead of the three previously described species.

1. Introduction

Traditionally, species delimitation in the plant kingdom has been based on morphological differences. However, the inclusion of molecular and ecological data in recent years has provided more evidence for delimiting species. Sites and Marshall (2003, 2004), in their synopsis of operational criteria for delimiting species boundaries, designated two broad categories: tree-based and non-tree-based methods. Among procedures for establishing species boundaries is the novel method proposed by Wiens and Penkrot (2002), which uses morphological and molecular data and tree-based and character-based analyses simultaneously. The WP method utilizes DNA haplotypes in parsimony analysis assuming a phylogeny of non-recombining haplotypes which may show the focal species to be either exclusive or not exclusive. The tree-based morphological analysis uses populations as terminals rather than individuals to avoid a biased treatment of the polymorphisms shared between populations as homoplasies rather than synapomorphies. Furthermore, the character-based analysis in the WP method involves finding diagnostic character states that represent differences among the putative species. The WP method considers strongly supported set of exclusive and geographically coherent populations to be potentially distinct species. In this paper this concept was expanded by performing niche modeling analyses. Current research considers niche modeling to be useful for identifying and diagnosing species (Raxworthy et al., 2007; Rissler and Apodaca, 2007; Stockman and Bond, 2007; Bond and Stockman, 2008) and for finding potential new species (Raxworthy et al., 2003). Niche modeling is able to provide evidence for geographic isolation among populations (based on either conserved or divergent ecological niches) and takes populations to be separately evolving lineages when gene flow is considered unlikely for the intervening geographic regions (Wiens and Graham, 2005).

A number of animal species were defined with the WP method (e.g., Hendrixson and Bond, 2005; Leavitt et al., 2007; Mulcahy, 2008) yet it has never been utilized with plants. It has been argued that polyploidy, asexual reproduction or hybridization are factors that solely affect evolutionary processes in plants (Rieseberg, 1997; Mable, 2004; Rieseberg and Willis, 2007; Silvertwon, 2008; Soltis et al., 2007). However, Rieseberg et al. (2006) have suggested that a lack of congruence between correspondence of plant and animal species could be in relation to these same factors but not by contemporary hybridization. Moreover, Rieseberg et al. (2006) generalized that plant species are more likely than animal species to represent reproductively independent lineages.

We selected the Neotropical bamboo *Otatea* (McClure and E.W. Sm) C. Calderón and Soderstr. as a test of the WP for delimiting plant species for a number of reasons. The first reason is that there is controversy on the recognition of two groups within *O. acuminata*, a variable species with the widest distribution in the genus. Initially *Otatea* was described with *Otatea acuminata* (Munro) C. Calderón and Soderstr. Later *Otatea aztecorum* was described but

* Corresponding author. Fax: +52 228 818 7809.
E-mail addresses: ruizsanchez.eduardo@gmail.com, eduardo.ruiz@posgrado.inecol.edu.mx (E. Ruiz-Sanchez), victoria.sosa@inecol.edu.mx (V. Sosa).

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it is now considered to be a subspecies of *O. acuminata* (subsp. *aztecorum*). Therefore *O. acuminata* is the focal species in our analyses. Two additional species were later described, *O. fimbriata* Soderstr. and *O. glauca* L.G. Clark and G. Cortés, and a new, still undescribed species from Chiapas is also known (Guzmán et al., 1984; Judziewicz et al., 1999; Clark and Cortés, 2004; Ruiz-Sanchez et al., 2008). What is more, our intensive fieldwork has discovered an elevated morphological variation in populations from previously unexplored areas, which have not yet been characterized.

The second reason to select this taxon, is that species in *Otatea* have allopatric distribution patterns and occupy different habitats, so that niche modeling could give new insights to identify and diagnose taxa. *Otatea acuminata* is endemic to Mexico, from Sonora along of the Pacific slopes to Chiapas and in central Mexico along the Transvolcanic Belt in tropical dry forest and xerophytic scrubs. *Otatea fimbriata* has a disjunct distribution, collected from three areas in Mexico and Central America, and one record from Colombia (Londoño and Clark, 1998) in dry pine–oak–juniper or even in dry tropical forests. *O. glauca* is endemic to Chiapas and only recollected from a single population, on slopes in the ecotone of tropical dry forests and oak forests. The undescribed species from Chiapas is currently only known from a single population in tropical dry forest.

The third reason for selecting *Otatea* is because its plants are monocarpic with a mass flowering. Populations usually flower for 2 or 3 years, and then the plants die. Herbarium records indicate that the cycles of mass flowering last 17–30 years. Thus it is difficult to find flowering plants to analyze floral characters. Molecular evidence for specimens lacking inflorescences might increase the number of informative characters used in defining species boundaries, together with vegetative morphological characters.

The main objective of this study is to set limits for the species belonging to *Otatea* using multiple data sets (molecular, morphological, and ecological). Four questions emerge from this objective: Do subspecies in *O. acuminata* merit species status? Could the disjunct population of *O. fimbriata* from Colombia be recognized as different? Can recently collected populations be assigned to previously described species or do they need to de described as new taxa? Does ecological niche modeling give new insights to identify and diagnose taxa? How many species should be recognized in the Neotropical bamboo genus *Otatea*?

2. Materials and methods

2.1. Taxon sampling

Individuals of *Otatea* were collected during 2005–2007 throughout the entire range of distribution of the genus (Fig. 1). *Olmeca recta* Soderstr., a taxon closely related to *Otatea*, was used as the outgroup (Ruiz-Sanchez et al., 2008). A total of 109 individuals from 28 populations were sampled with 3–12 individuals per population (Fig. 1, Table 1). Fresh leaves were collected from each individual and dried in silica (Chase and Hills, 1991) and the vouchers were deposited at XAL.

2.2. Morphological data set

The morphological matrix included 54 characters (40 vegetative, six from synflorescences and flowers and eight leaf micromorphological characters (Appendix 1; data matrix in Appendix 2). Character selection was based on the list and illustrations by L. Clark in "Bamboo Biodiversity" (http://www.eeob.iastate.edu/research/bamboo) and on Londoño and Clark (2002). Characters were scored by examining live material and herbarium specimens. Vouchers and examined specimens are also listed in Appendix 3.

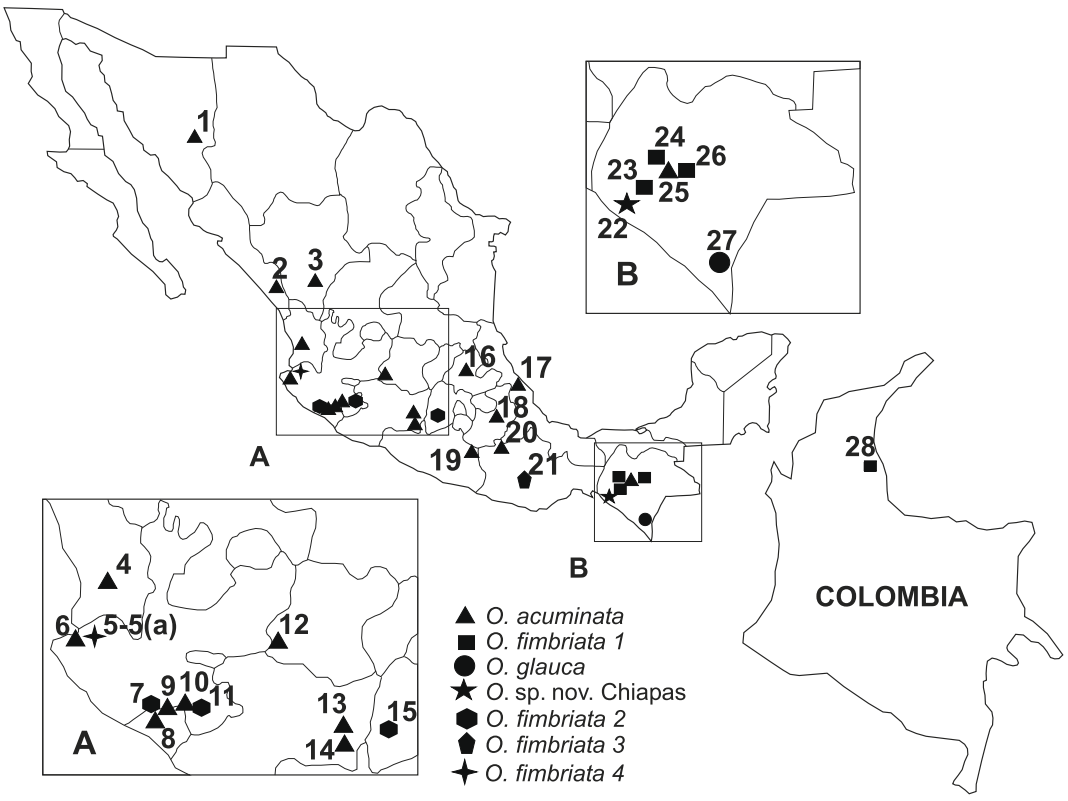

Fig. 1. Distribution and sampling localities of the previously recognized species of *Otatea*. Numbers correspond to localities in Table 1. (A and B) Details of localities in central Mexico and Chiapas, respectively.

Table 1
Study populations and their haplotypes. *n* = number of individuals sampled for cpDNA markers, and numbers below ITS are the individuals sampled to this marker.

Population	Locality	Lat.	Long.	Altitude (m a.s.l.)	*n*	Species	Haplotype	ITS
1	Sonora	28 21	109 15	430	3	*O. acuminata*	F	2
2	Sinaloa	23 26	105 50	1205	3	*O. acuminata*	F	1
3	Durango	23 29	104 26	1652	3	*O. acuminata*	F	1
4	Nayarit	21 35	104 56	758	3	*O. acuminata*	F	1
5	Jalisco	20 42	104 53	1506	3	*O. fimbriata*	F	3
5a	Jalisco	20 43	104 53	1393	–	*O. fimbriata*	–	–
6	Jalisco	20 27	105 17	425	3	*O. acuminata*	F	1
7	Colima	19 24	103 51	1710	5	*O. fimbriata*	F	4
8	Colima	19 22	103 51	1436	4	*O. acuminata*	A	3
9	Colima	19 27	103 43	1146	3	*O. acuminata*	A	3
10	Jalisco	19 26	103 28	922	3	*O. acuminata*	F	1
11	Jalisco	19 26	103 21	1181	5	*O. fimbriata*	F	3
12	Guanajuato	20 32	101 53	1917	5	*O. acuminata*	F	1
13	Michoacán	19 14	100 47	650	3	*O. acuminata*	F	1
14	Michoacán	18 50	100 53	710	5	*O. acuminata*	G	1
15	Edo. Mexico	19 03	100 01	1840	10	*O. fimbriata*	E–F	4
16	Hidalgo	20 38	98 59	1735	3	*O. acuminata*	D	2
17	Veracruz	19 21	96 51	667	3	*O. acuminata*	C	3
18	Puebla	18 36	97 55	1606	3	*O. acuminata*	F	1
19	Guerrero	17 44	98 35	1020	3	*O. acuminata*	F	1
20	Oaxaca	18 01	97 20	1967	3	*O. acuminata*	F	1
21	Oaxaca	16 37	96 54	1525	3	*O. fimbriata*	B	3
22	Chiapas	16 02	93 35	843	3	*O.* sp. nov. Chiapas	I–J	3
23	Chiapas	16 28	93 13	1030	3	*O. fimbriata*	B	1
24	Chiapas	16 45	92 56	1086	3	*O. fimbriata*	B	1
25	Chiapas	16 42	92 54	1017	3	*O. acuminata*	B	3
26	Chiapas	16 41	92 51	1320	3	*O. fimbriata*	B	1
27	Chiapas	15 19	92 19	1200	3	*O. glauca*	H	2
28	N Santander	08 10	73 18	1230	12	*O. fimbriata*	B	8

2.3. Molecular data set

2.3.1. DNA extraction, amplification, and sequencing

DNA was isolated using the modified 2X CTAB method (Cota-Sánchez et al., 2006). Three chloroplast regions (*atpF-atpH*, *psbK-psbI*, *trnL-rpl32*) were used; the first two were amplified and sequenced using primers and protocols by Lahaye et al. (2008) and the latter following Shaw et al. (2007). In addition, the nuclear DNA region ITS, using the ITS5 and ITS4 primers of White et al. (1990), following the protocol of Shrestha et al. (2003) was also sequenced. Amplified products and DNA were purified using the QIAquick PCR purification kit (Qiagen, California, USA) following the protocols provided by the manufacturer. Clean products were sequenced using the *Taq* BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, USA) with an ABI 310 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, USA). Electropherograms were edited and assembled using Sequencher 4.1 (Gene Codes, Ann. Arbor, MI). Sequences were manually aligned with Se–Al v. 2.0a11 (Rambaut, 2002).

2.4. Phylogenetic analyses

Two sets of phylogenetic analyses were conducted, molecular and morphological. With molecular data, initially two parsimony (MP) analyses were conducted, one based on the combined chloroplast *atpF-atpH*, *psbK-psbI*, *trnL-rpl32* intergenic loci and a second with the nuclear ITS. Finally a combined molecular analysis with chloroplast and nuclear DNA loci was performed.

Parsimony analyses were run in TNT (Goloboff et al., 2003) using a new technology search approach, the ratchet algorithm with 200 iterations. Parsimony bootstrapping support for internal branches was estimated with 1000 replicates using TBR branch swapping, with 10 random entry orders saving one tree per replicate. The potential incongruence of the molecular and morphological data sets was tested using the incongruence length difference (ILD) test of Farris et al. (1994) as implemented in WinClada (Nixon, 1999–2002).

In the morphological phylogenetic analysis the 28 populations of *Otatea* were treated as terminal units (Fig. 1, Table 1). The data matrix included 54 characters, two of which were autapomorphic (Appendix 3), and it was constructed with WinClada (Nixon, 1999–2002). Parsimony and bootstrap support analyses were run in TNT (Goloboff et al., 2003) with the same settings indicated for the molecular data. Bremer support (Bremer, 1994) was calculated using the BS5 option of Nona (Goloboff, 1999) on 10,000 trees held in memory.

A statistical parsimony network was obtained with the program TCS v.1.21 (Clement et al., 2000), to understand the genotype relationships of every individual. The network was constructed using concatenated sequences (chloroplast and nuclear DNA sequences).

2.5. Morphological character-based species delimitation

The character-based approach was implemented by comparing the frequencies of qualitative characters and the range of trait values for quantitative continuous and meristic characters across all populations to search for potentially diagnostic characters. Characters were considered to diagnose a species or a set of populations if they were invariant for alternative character states or showed no overlap in trait values as indicated by Wiens and Penkrot (2002).

2.6. Ecological niche modeling

To determine niche dimensions, niche differences and geographic predictions in the studied populations of *Otatea*, two algorithms that vary in their predictive performance were used (Elith et al., 2006); GARP (DesktopGarp v 1.1.6; http://nhm.ku.edu/desktopgarp/index.html) and Maxent v 3.2.1 (Phillips et al., 2006). The use of niche modeling can help differentiating between two hypotheses (niche conservatism vs. niche divergence). Niche conservatism promotes allopatric speciation, by limiting dispersal between populations (e.g., Wiens and Graham, 2005). Niche divergence indicates adaptation to different ecological conditions in

allopatric or parapatric populations accelerating the evolution of reproductive isolation (Kozak et al., 2008).

A total of 134 georeferenced records were compiled, including 102 for *Otatea acuminata* and 37 for *O. fimbriata*. However, *O. glauca* and *O.* sp. nov. Chiapas have each been recorded in only a single locality. Georeferenced locality data were obtained during the fieldwork of this project and from herbarium specimens from the following herbaria: ENCB, F, IBUG, IEB, ISC, MEXU, MO, NY, US and XAL. The dataset was modeled with 19 standard bioclimatic variables derived from modern temperature and precipitation data from WorldClim 1.4 (Hijmans et al., 2005) with a resolution of 1 km^2.

Maxent creates species distribution models (DMs) by combining presence-only data with ecological layers using a statistical approach known as maximum entropy. The maximum entropy approach estimates a species' environmental niche by finding a probability distribution that is based on a distribution of maximum entropy (Rissler and Apodaca, 2007). Following Phillips et al. (2006), we used the default modeling parameters for all species with a logistic output. Binary maps (predicted presence or absence) were created from the Maxent-generated niche distribution models using the lowest presence threshold value (LTP) (Pearson et al., 2007; Stockman and Bond, 2007; Bond and Stockman, 2008).

The genetic algorithm for rule-set prediction (GARP) (Stockwell and Noble, 1992; Stockwell and Peters, 1999) like Maxent, reconstructs the potential distribution of species with an evolutionary computing genetic algorithm to search for a non-random association between environmental variables and known occurrences of species, as contrasted with environmental factors across the study area (Stockwell and Peters, 1999). GARP was run for species with more than 25 records with the parameters: 50% for training, 50% for testing, runs = 100, convergence limit = 0.01, iterations = 1000. "Best subsets" omission measure = extrinsic, omission threshold = hard and 10% omission, total models under hard omission threshold = 20, commission threshold = 50%). For species with fewer than 20 records GARP analysis was run with the same parameters except for 100% for training and omission measure = intrinsic. Both procedures generated 10 best models. The geographic predictions (binary predictions, 0 = absence, 1 = presence) of the 10 best models were averaged to provide a summary of potential geographic distributions (Anderson et al., 2003). *Otatea glauca* and *O.* sp. nov. Chiapas were not considered for ecological niche modeling because there are fewer than five records for each. The resultant Maxent and GARP ASCII file was converted to raster format using ArcView GIS 3.2.

To evaluate ecological interchangeability, two evaluations were performed (Stockman and Bond, 2007; Bond and Stockman, 2008). From the closely related lineages we estimated the degree of overlap following Barraclough and Vogler (2000) from the DMs generated by Maxent and GARP. Firstly, from the closed related lineages the degree and significance of overlap between closely related lineages was estimated with D-NOVL v 1.3 (Stockman et al., 2008). This program uses a Monte Carlo algorithm that creates a null model to determine the probability distribution of the degree of overlap for DMs of known sizes (Stockman and Bond, 2007). We then used standard methods of statistical inference to evaluate the amount of overlap observed. The null hypothesis that the DMs are randomly distributed is rejected if the observed overlap has a probability <0.05. Lineages whose DMs are completely or largely overlapping are considered to be ecologically interchangeable (Stockman and Bond, 2007). For each closed related lineage we performed 1000 simulations following Stockman et al. (2008). Secondly, a PCA was performed using the extracted values of 19 climate variables (WorldClim 1.4; Hijmans et al., 2005) for each unique locality of each clade to examine the overall level of divergence in environmental space among the extant taxa. We then quantified how pairs of sister taxa overlapped in environmental space using the environmental variables. A MANOVA (multivariate analysis of variance) of PC scores was performed to test for significant differences among the PC scores of closely related lineages. The *F*-statistic was reported, as well as a test of between-subject effects to determine which PCs account for significance in the overall test (Graham et al., 2004; Stockman and Bond, 2007).

3. Results

3.1. DNA haplotype phylogenetic analyses

Sequence lengths for the three chloroplast *atpF-atpH*, *psbK-psbI* and *trnL-rpl32* intergenic loci were 622, 438 and 847 base pairs (bp), respectively, and total length was 1907 bp. All sequences were deposited in GenBank (accession numbers; *atpF-atpH*; FJ483849-FJ483862; *psbK-psbI*: FJ483863-FJ483876; *rpl32-trnL*: FJ483877-FJ483888); twenty parsimony-informative characters, 36 variable sites and 12 haplotypes were found (Table 1). Sequence length for nuclear ITS, was 587–593 bp; 42 parsimony-informative characters, 49 variable sites and 33 haplotypes were found (Accession Nos. GQ384308–CQ384341).

The cpDNA data resulted in a single most parsimonious tree (MPT) (L = 21, CI = 0.95, RI = 0.99) (Fig. 2). The ITS resulted in eight MPT (L = 0.95, CI = 0.64, RI = 0.88), strict consensus is shown in (Fig. 2). The combined data set resulted in five MPT (L = 124, CI = 0.65, RI = 0.91) strict consensus is shown in Fig. 2. The ILD test revealed no incongruence between the chloroplast and the nuclear datasets ($P = 0.1517$). The single chloroplast MPT shows that the haplotypes of *Otatea acuminata* and *O. fimbriata* are not exclusive and formed two different supported clades. Individuals of the two subspecies of *O. acuminata* did not form groups. Individuals of *Otatea glauca* and *O.* sp. nov. Chiapas are combined and only 2 individuals of the populations of *O.* sp. nov. Chiapas formed an exclusive group (Fig. 2). ITS strict consensus tree shows similar topology to the chloroplast single MPT (Fig. 2). Strict consensus topology of the combined data matrix is similar to the chloroplast MPT, showing besides the two main clades an additional supported clade formed by individuals of *O. glauca* and *O.* sp. nov. Chiapas (Fig. 2).

3.2. Statistical parsimony network

The network with the 108 *Otatea* individuals resulted in 34 unique compound (chloroplast + nuclear) genotypes grouped in a single network (Fig. 3). Seventeen genotypes were found in *O. acuminata*, 12 in *O. fimbriata*, 2 in *O. glauca*, and 1 for *O.* sp. nov. Chiapas, respectively (Fig. 3). The biggest outgroup probability was for population 28 of *O. fimbriata* from Colombia.

3.3. Morphological phylogeny

Parsimony analysis of morphological data resulted in a single most parsimonious tree (MPT) (L = 179, CI = 0.47 and RI = 0.76), shown in Fig. 4. MPT displays populations of *Otatea acuminata* (B = 4) (Bootstrap support = BS and Bremer support = B) and *O. fimbriata* in their own clades, as well as populations of *O. glauca* and the single population (22) of *O.* sp. nov. Chiapas. Populations from the subspecies of *O. acuminata* are combined in the same clade. Three main clades were retrieved, a first clade formed by populations of *O. glauca* and *O.* sp. nov. Chiapas; a second clade formed by populations of *O. fimbriata* with four subclades from the populations of *O. fimbriata* 1, 2, 3 and 4; the third clade was formed by populations of the two subspecies of *O. acuminata* (Fig. 4).

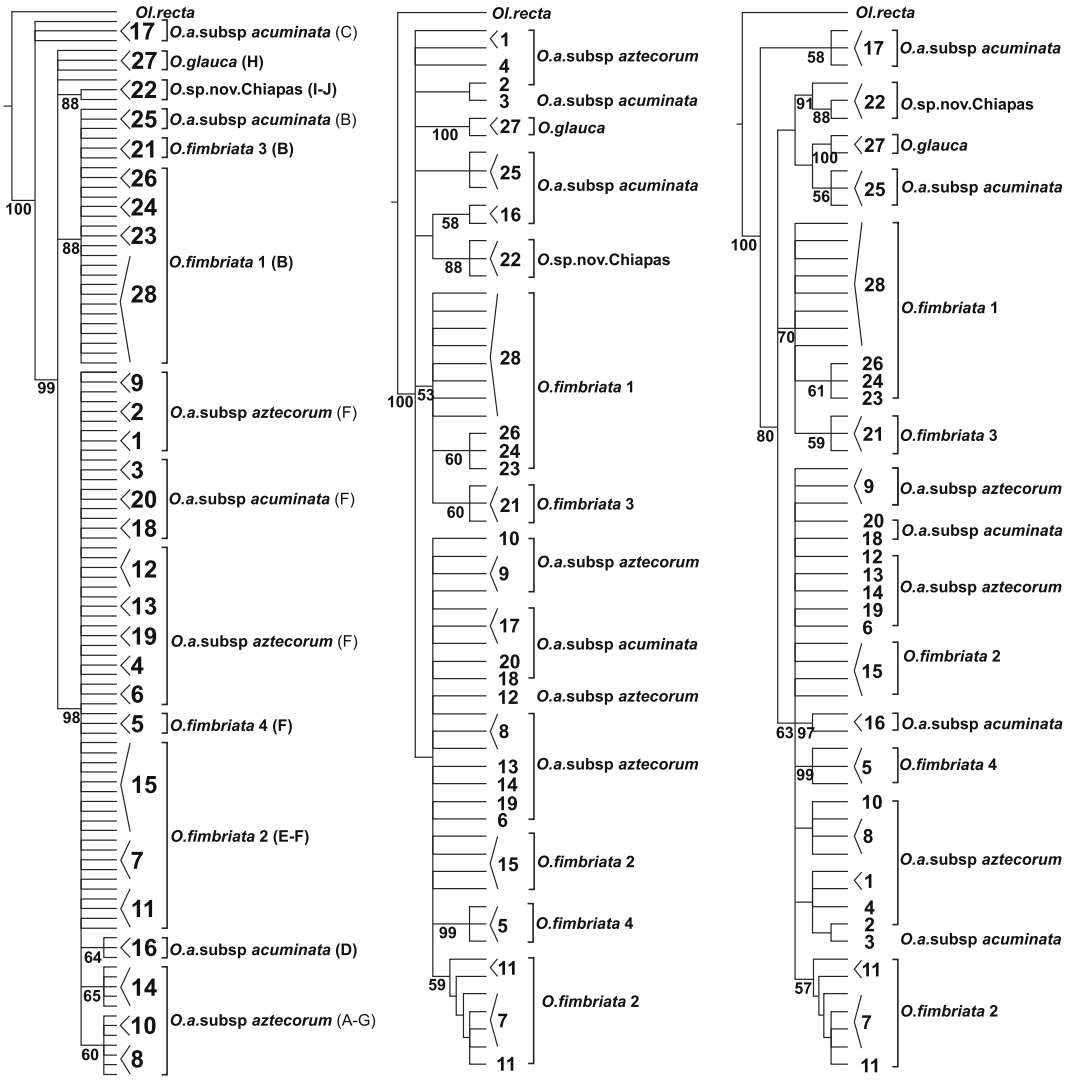

Fig. 2. MPT on chloroplast DNA haplotypes for *Otatea* (left). Population numbers and haplotypes in parenthesis correspond to Table 1. Number below branches indicates Bootstrap values. Strict consensus from parsimony analysis of the ITS (center) and combined cpDNA-ITS (right). Populations numbers correspond to Table 1. Number below branches indicates Bootstrap values. New species retrieved by the morphological tree are indicated.

3.4. Character-based species delimitation

Otatea acuminata is the most morphologically variable species with a widespread distribution (Fig. 1) and is recognized by a single diagnostic character: the lack of oral setae in foliar leaves (Char. 28) and the two subspecies can not be recognized based on morphological characters. An inclusive clade was formed by populations of *O. fimbriata* comprising four subclades. The first subclade, which includes the population collected in the type locality (as *Otatea fimbriata* 1 in Figs. 1–5) is distributed from Chiapas, through Central America to South America. It is recognized by three diagnostic characters: nodal line dipping slightly below the buds, brown foliar oral setae, and a patch of brown of cilia on the abaxial foliar surface. The second subclade, inlcuding populations from the Transvolcanic Belt (as *Otatea fimbriata* 2 in Figs. 1–5) has five diagnostic characters: an extravaginal branching pattern, branch complement with one or two divergent branches, a glabrous sheath apex, foliar oral setae connate at the basal third or more, and a patch of yellow cilia on the abaxial foliar surface. The third subclade, comprises a single population from Oaxaca (as *Otatea fimbriata* 3 in Figs. 1–4) has two diagnostic characters: purple foliar oral setae with a length of 6 mm. Lastly, the fourth sublcade included as well an only population from Jalisco (as *Otatea fimbriata* 4 in Figs. 1–5) with three diagnostic characters: papyraceous foliar oral setae, white culm and foliage leaf oral setae. *O. glauca* from Chiapas has three diagnostic characters: thin culm walls and glabrous foliar oral setae. *O.* sp. nov. Chiapas has two diagnostic characters: fimbriate culm leaf bases and straight fimbriae of foliar leaves.

3.5. Ecological niche models and PCA/MANOVA

Based on the morphological tree phylogeny, which was coherent with geographic distribution (Figs. 1 and 4), GARP and Maxent predictive models were produced based on 19 environmental variables (Table 2) for only the four entities with more than five records (Pearson et al., 2007). These species were *Otatea acuminata*, *O. fimbriata* 1, *O. fimbriata* 2 and *O. fimbriata* 4, none of which are sister taxa (Fig. 4). Distribution models (DMs) of *O. acuminata* (Fig. 5a GARP and 5b Maxent; LTP = 11%) were based on 103 samples, the highest number of records with the widest distribution in Mexico. GARP and Maxent DMs for *O. acuminata* (Fig. 5a) found the areas of predicted occurrence to be similar. The DMs of *O. fimbriata* 1(Fig. 5c GARP and 5d Maxent; LTP = 57%) were based on 19

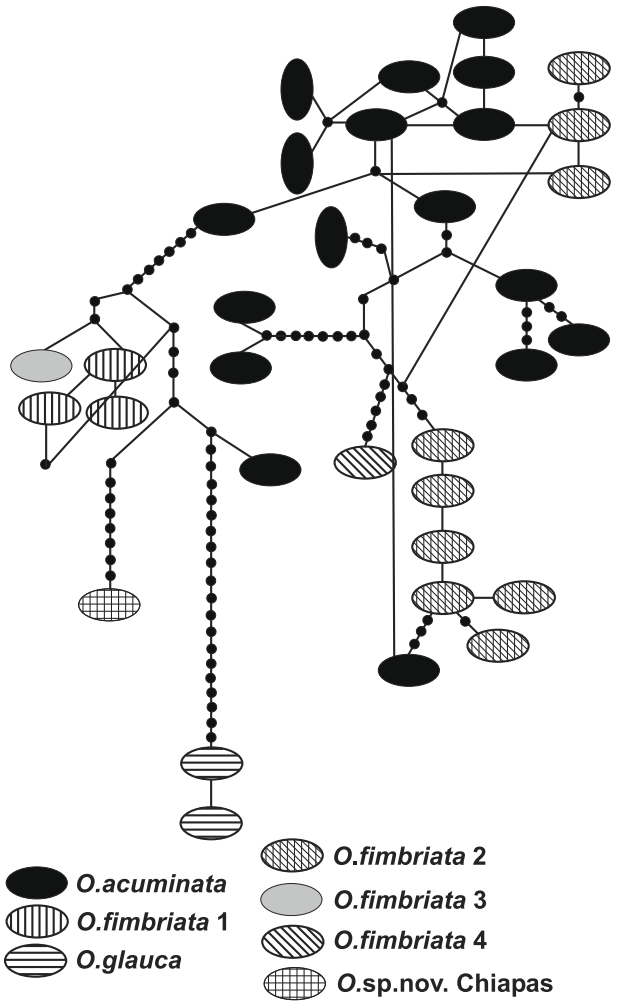

Fig. 3. Statistical parsimony network compound cpDNA-ITS genotypes.

records and both algorithms predicted almost the same DMs, with suitable areas in Oaxaca, Guerrero, Michoacán, Estado de México, Colima and Jalisco from Mexico and Guatemala, Nicaragua and Venezuela. DMs of *O. fimbriata* 2 (Fig. 5e GARP and 5f Maxent; LTP = 58%) were based on 10 records. The GARP (Fig. 5e) prediction was better than that of Maxent (Fig. 5f), because it excluded a record from Nayarit. The DMs of *O. fimbriata* 4 (Fig. 5g GARP and 5 h Maxent; LTP = 56%) were based on seven records. In this case the GARP prediction was better than that of Maxent, because the latter overpredicted suitable areas in Nayarit, Jalisco, Michoacán, Estado de Mexico and D.F. GARP DMs for *O. fimbriata* 2 and *O. fimbriata* 4, with 10 or fewer records gave better predictive models than Maxent did (Fig. 5e–h).

The PCA based on 19 climate variables found that PC1 = 33%; PC2 = 30%; PC3 = 13.7% and PC4 = 10.3% explained almost 87% of the variability. Table 2 shows that seven temperature variables load negatively on PC1, one temperature and three precipitation variables load on PC2, one temperature and one precipitation variable load on PC3, and finally two precipitation variables load on PC4.

The PCA/MANOVAs of the lineages recognized from the morphological phylogeny gave the following results. The comparison between *Otatea glauca* with *O.* sp. nov. Chiapas was not performed because of the low number of sample records for the two species. However, a statistically significant difference between PC1 and PC4 ($P = 0.007$, $P = 0.01$) was found for these groups, suggesting that they are found in areas with different precipitation and temperature regimes. The overall MANOVA shows a statistically significant difference between *O. fimbriata* 3 and *O. fimbriata* 4 ($F_{1,8} = 841.7$, $P < 0.0001$). This difference occurs along PC2 ($F = 18.7$, $P = 0.004$), PC3 ($F = 42.07$, $P = 0.0006$) and PC4 ($F = 62.7$, $P = 0.0002$), suggesting that these two entities are found in areas with different amount of precipitation, but similar temperatures. The comparison of the groups formed by populations from *O. fimbriata* 3 and 4 with populations of *O. fimbriata* 2 showed no statistically significant differences ($F_{1,16} = 1.21$, $P = 0.3526$), suggesting that the three entities are found in areas with similar temperature and precipitation. *O. fimbriata* 1 and (*O.* fimbriata 2, 3 and 4) were significantly different ($F_{1,35} = 23.89$, $P < 0.0001$). This difference occurs along PC1 ($F = 6.19$, $P < 0.018$), PC2 ($F = 43.09$, $P < 0.0001$) and PC4 ($F = 14.93$, $P = 0.0005$), suggesting that the entities are found in areas with different precipitation and temperature regimes.

The ecological interchangeability models produced by GARP and Maxent revealed that *Otatea fimbriata* 4 and *O. fimbriata* 2 showed a large degree of overlap (GARP = 70.8% and Maxent = 65.4%) and the probability of detecting overlap if the ranges were randomly distributed was <0.05, suggesting that these two clades display little ecological divergence. This agreed with the MANOVA results. GARP and Maxent DMs for *O. fimbriata* 1 with *O. fimbriata* 2 and 4 had minimal or no overlap. *O. fimbriata* 1 with *O.* fimbriata 2 (GARP = 8.2% and Maxent = 8.3%) and *O. fimbriata* 1 with *O. fimbriata* 4 (GARP = 0% and Maxent = 3.9%) were consistent with a random distribution ($P > 0.05$), therefore there is no ecological interchangeability between *O. fimbriata* 1 with either *O. fimbriata* 2 or *O. fimbriata* 4 and they have divergent ecological niches. In conclusion, the results of the above combinations indicate that populations of *O. fimbriata* 1 occur in areas with different precipitation and temperature regimes.

4. Discussion

4.1. Molecular analyses

Instead of chloroplast loci such as *trnD-trnT, trnC-rpoB, rps16-trnQ,* and the *rpl16* intron, which are currently being utilized in the Bamboo Phylogeny Project (http://www.eeob.iastate.edu/research/bamboo), we sequenced *atpF-atpH*, *psbK-psbI*, because more variation in a large number of angiosperms has been reported for them (Lahaye et al., 2008), and we used the spacer *trnL-rpl32* because it is one of the most variable according to Shaw et al. (2007). The loci of the Bamboo Phylogeny Project were selected to determine the relationships of groups at higher taxonomic levels. The nuclear ITS has been sequenced for temperate woody bamboos and we used it as well (Hodkinson et al., 2000; Guo et al., 2001, 2002; Guo and Li, 2004; Peng et al., 2008; Sun et al., 2005; Yang et al., 2008).

Trees retrieved by molecular analyses were mostly unresolved. Low substitution rates have been found in Bambusoideae phylogenetic analyses that used different DNA markers (Kelchner and Clark, 1997; Hodkinson et al., 2000; Guo et al., 2001, 2002; Guo and Li, 2004; Sun et al., 2005; Yang et al., 2008; Bouchenak-Khelladi et al., 2008; Peng et al., 2008; Ruiz-Sanchez et al., 2008; Sungkaew et al., 2008). In spite of the fact that we selected these previously reported and variable markers, they had low numbers of informative molecular characters in the phylogenetic analyses for *Otatea*.

Tree topologies retrieved by the molecular analyses were similar and individuals of *Otatea glauca* and *O.* sp. nov. Chiapas were the only ones to form supported clades. However, according to the WP method, molecular- and morphological tree-based, character-based analysis and geographic coherence should be taken into account to define species, so decisions for *Otatea* were taken based in all evidence.

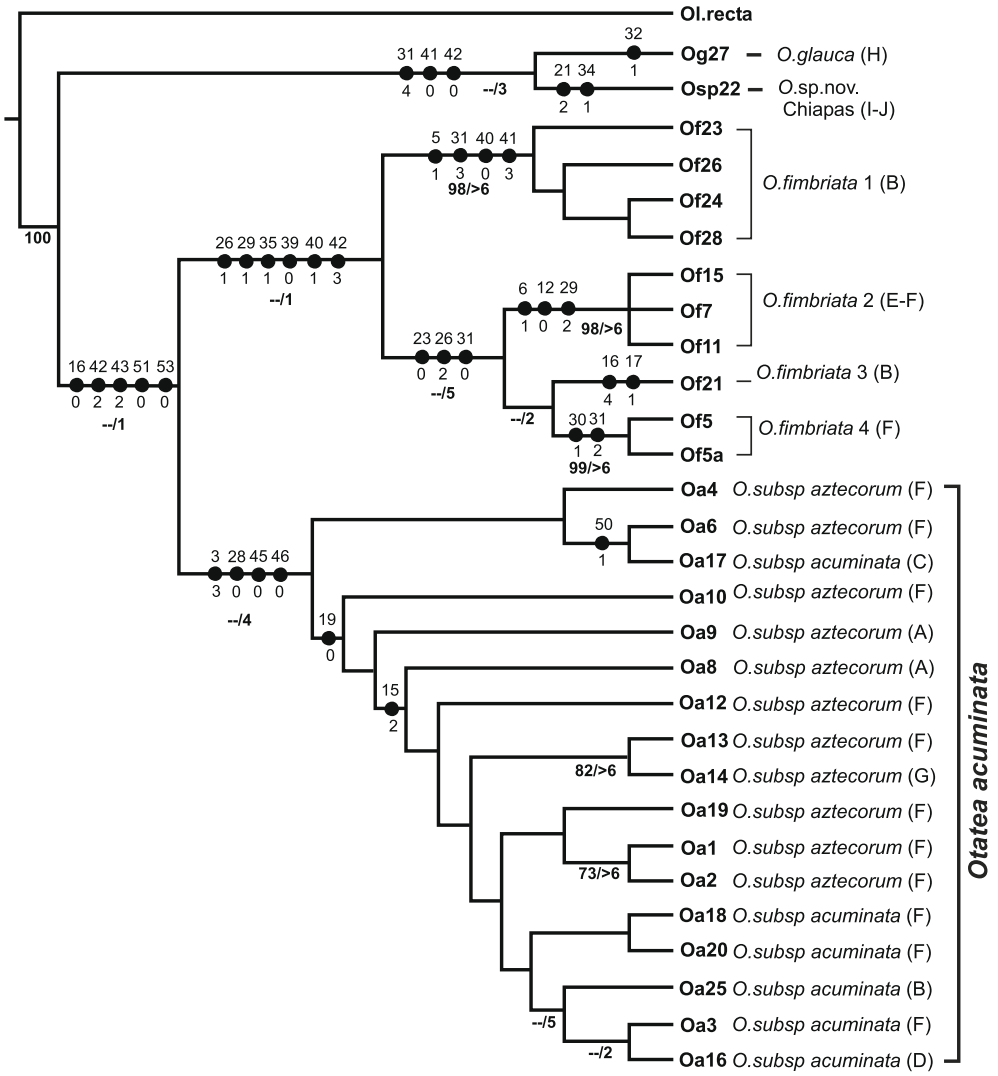

Fig. 4. The single most parsimonious tree retrieved from the morphological data set (L = 179; CI = 47; RI = 76). Population numbers and haplotypes in parenthesis correspond to Table 1. Black circles indicate synapomorphies, numbers above and below the circles indicate character number and character state, respectively. Numbers below the branches indicate Bootstrap and Bremer support. Ol.recta, *Olmeca recta*; Oa, *Otatea acuminata*; Of, *Otatea fimbriata*; Og = *Otatea glauca*.

The statistical parsimony network identified a single network, implying that both markers (chloroplast and nuclear) have congruent histories with low global homoplasy levels (Gómez-Zurita and Vogler, 2006). The network exhibits some reticulation, particularly between genotypes of *Otatea acuminata* and *O. fimbriata* 2.

4.2. Morphological analyses

Morphological parsimony retrieved only three well supported clades (*Otatea fimbriata* subclades 1, 2, 3 and 4). Character-based analysis recognized seven identities, which possess the diagnostic character states indicated above. These are: *O. acuminata* (including subsp. *aztecorum*), *O. fimbriata* 1 (including the Colombian population), *O. glauca*, and four putative new species: *O. fimbriata* 2, 3 and 4 and the population *O.* sp. nov. from Chiapas. Diagnostic characters were all vegetative. In *Otatea*, in contrast to many other Neotropical bamboo genera, there appear to be relatively few good characters in the floral structures even when they are present to distinguish reliably among its species, but flowering specimens were not known for some of our populations. Traditionally, a number of morphological characters have been used to differentiate taxa in bamboos (Londoño and Clark, 1998, 2002; Judziewicz et al., 1999). The vegetative attribute of a branch complement with three subequal branches per node is the character that has been used to distinguish *Otatea* from the other genera in subtribe Guaduinae (Guzmán et al., 1984; Londoño and Clark, 1998, 2002; Judziewicz et al., 1999; Ruiz-Sanchez et al., 2008). An additional character is the presence of oral setae at the apex of the culm and foliage leaf sheaths (Ruiz-Sanchez et al., 2008). These characters vary among the taxa recognized by our analyses, but differences in habitats (types of vegetation) and an allopatric distribution could be responsible for this variation.

The focal species in our study, *Otatea acuminata*, displayed an elevated morphological variation. Diameter of stems was variable. The subsp. *aztecorum* was recognized by having thick stems, more than 3 cm in diameter. Also, pubescence on abaxial sheath surface was another character for differentiating *O. acuminata* subsp. *aztecorum* from subsp. *acuminata*. Yet our results did not find these characters supporting groups of populations in the tree-based nor as diagnostic in the character-based analyses. Oral setae on culm leaves were either present or lacking in populations of *O. acuminata*, but they were always absent from foliar leaves, and this was a diagnostic attribute according to the character-based analysis. In contrast to vegetative attributes, floral characters were not

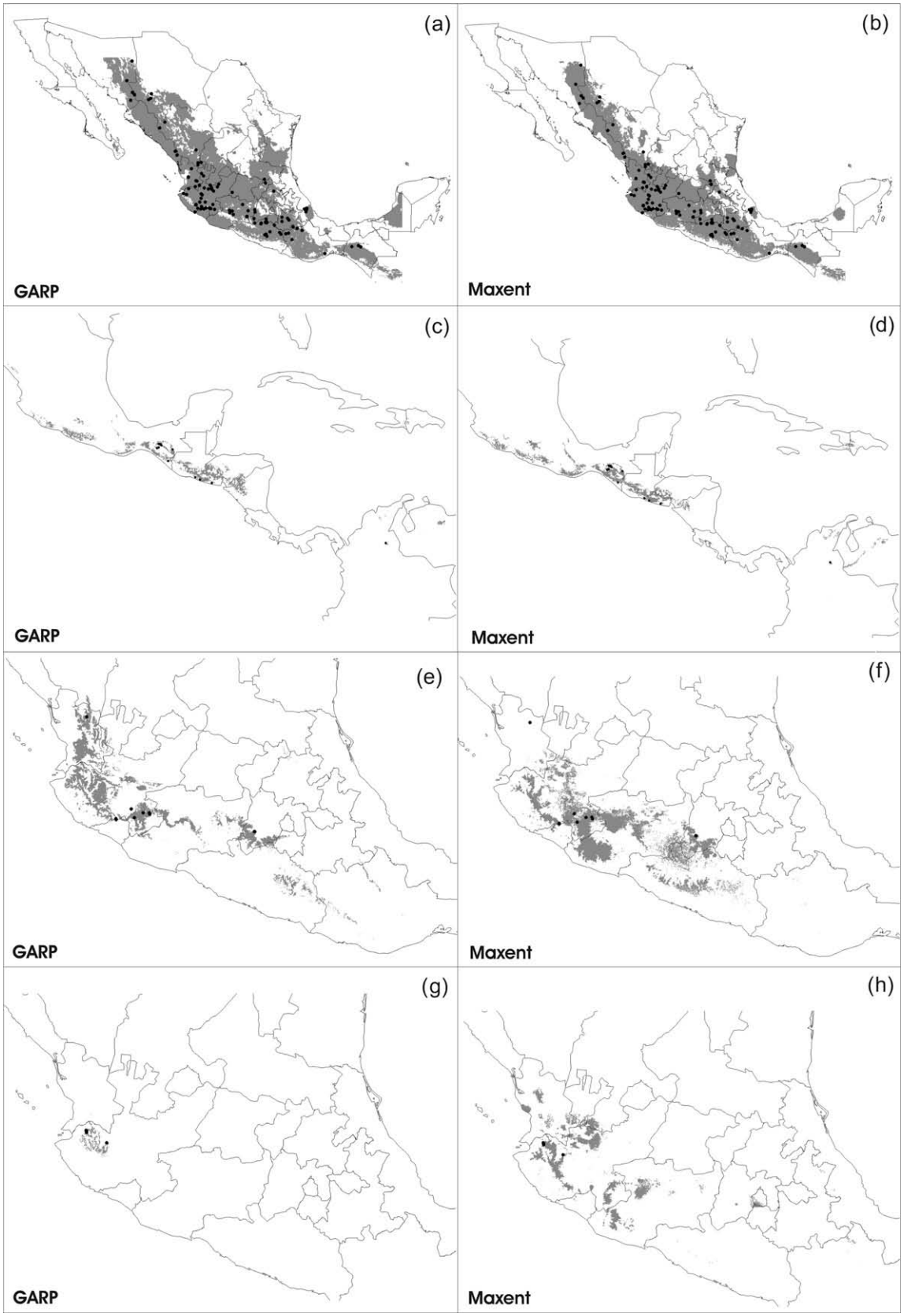

Fig. 5. GARP and Maxent niche-based distribution models for: (a and b) *O. acuminata* maps, (c and d) *O. fimbriata* 1, (e and f) *O. fimbriata* 2 maps, (g and h) *O. fimbriata* 4.

variable in populations of this species. Qualitative characters, such as color of oral setae or cilia on the abaxial foliar surface or pubescence on sheath apex that resulted diagnostic for entities such as *O. fimbriata* 1, 2 and 4 were observed from both wild plants and from new shoots of cultivated plants in the Clavijero Botanical Garden.

Table 2
Nineteen climate variables used in GARP and MAXENT analysis and PCA loadings for the four principal components. Values in bold indicate higher loadings.

Climate variable	PC1	PC2	PC3	PC4
Annual mean temperature	**−0.975320**	−0.143425	0.032460	−0.145066
Mean diurnal range	−0.112774	0.753569	0.194854	0.180583
Isothermality	−0.023475	−0.685331	−0.514544	0.002727
Temperature seasonality	0.027975	0.728738	**0.623226**	−0.033811
Max temperature of warmest month	−0.889275	0.337008	0.241534	−0.078595
Min temperature of coldest month	−0.769070	−0.563384	−0.199986	−0.190892
Temperature annual range	−0.070883	**0.860841**	0.418231	0.113930
Mean temperature of wettest quarter	**−0.906086**	0.119898	0.314140	−0.133796
Mean temperature of driest quarter	**−0.957026**	−0.130334	0.071173	−0.029564
Mean temperature of warmest quarter	**−0.946860**	0.124293	0.236632	−0.149047
Mean temperature of coldest quarter	**−0.882372**	−0.393891	−0.197232	−0.114134
Annual precipitation	−0.002388	**−0.906328**	0.282775	0.167807
Precipitation of wettest month	−0.051603	**−0.764931**	0.397377	0.464326
Precipitation of driest month	0.324830	−0.423210	0.511135	**−0.579483**
Precipitation seasonality	−0.431499	0.301014	−0.062085	**0.746604**
Precipitation of wettest quarter	−0.118377	**−0.807392**	0.305036	0.446147
Precipitation of driest quarter	0.373867	−0.435252	0.549025	−0.538394
Precipitation of warmest quarter	0.152462	−0.362475	**0.571041**	0.423750
Precipitation of coldest quarter	0.191493	−0.322610	0.468286	0.021680

4.3. Ecological niche

The principle for projecting ecological niche is that adaptation to different climate conditions in allopatric or parapatric populations might play an important role in speciation by driving phenotypic divergences and accelerating the evolution of reproductive isolation (Kozak et al., 2008). Niche divergence implies that there is no ecological interchangeability while niche conservatism implies the contrary (Graham et al., 2004; Jakob et al., 2007; McGuire et al., 2007; Rissler and Apodaca, 2007; Stockman and Bond, 2007; Bond and Stockman, 2008). The results of GARP, MAXENT and PCA/MANOVAS suggest niche divergence for *Otatea fimbriata* 1, 2, 3 and 4. *Otatea fimbriata* 4 and *O. fimbriata* 3 have divergent ecological niches. Morphological results indicate that the allopatric sister species, *O. fimbriata* 4 and *O. fimbriata* 3, are divergent in ecological niches. The species from Jalisco (*O. fimbriata* 4) grows in pine–oak humid forests or cloud forests and the species from Oaxaca (*O. fimbriata* 3) grows in pine–juniper–oak forests with differences in precipitation. Populations of *Otatea acuminata* resulted the sister group to a clade formed by populations of *O. fimbriata* 1, 2, 3 and 4 in the morphological tree. Ecological niche models are projected for sister taxa, thus it was not possible to consider *O. acuminata*, our focal species, in these projections. Moreover, populations of *O. acuminata* are found from tropical dry forest to even drier habitats, like xerophytic scrubs. In addition this species is found in altitudes from 400 to 2000 m.

4.4. Conflicting results from molecular and morphological analyses

Molecular and morphological analyses retrieved trees with conflicting topologies. Probable causes of incongruence between molecular and morphological data sets include processes like lineage sorting of ancestral polymorphisms, paralogy, lateral gene transfer and hybridization (Brower et al., 1996; Maddison, 1997). Recent research on plants (Jakob and Blattner, 2006; Bänfer et al., 2006; Flagel et al., 2008; van der Niet and Linder, 2008) and animals (Pollard et al., 2006; Carstens and Knowles, 2007a,b; Leaché and Mulcahy, 2007; McGuire et al., 2007; Gray et al., 2008; Trewick, 2008) has found that incomplete lineage sorting is often one of the causes of incongruence between gene and species trees. Furthermore, the effect of lineage sorting persists regardless of whether more and more regions within a genome are added to the analyses or not (Hudson and Coyne, 2002; Degnan and Rosenberg, 2006; Knowles and Carstens, 2007). Recently derived species may reflect the incongruence between species boundaries inferred with genetic markers and the boundaries inferred with morphology (Knowles and Carstens, 2007), which could be the case in *Otatea*.

As mentioned above, in plants, hybridization processess can also cause discordance between morphological and molecular data (e.g., Lihová et al., 2007; Pan et al., 2007). In Bambusoideae natural hybridization has been reported in the Neotropical genus *Chusquea* (Clark et al., 1989) and in a Japanese lineage formed by genera such as *Pleioblastus, Sasamorpha, Phyllostachys*, and *Sasa*, representing intergeneric hybrids (Triplett and Clark, 2009). However, hybridization or introgression are possible explanations for the observed patterns, but they cannot be ruled out or confirmed by the methods employed in this study. These processes could be understood by utilizing molecular markers such as microsatellites or AFLP's.

5. Conclusions

Trees recovered by molecular and morphological analyses retrieved two contrasting hypotheses. Molecular analyses indicated that species of *Otatea* have diverged recently with gene exchange, hybridization or incomplete lineage sorting. Therefore, we base our conclusions on the tree-based and character-based morphological analyses as suggested by the WP methodology, recognizing four new species, three of them segregated from *Otatea fimbriata* plus three currently recognized in *Otatea*, each with fixed diagnostic characters. Moreover ecological niche modeling agreed that niche divergence could be one of the causes of speciation, because allopatric groups of populations were separated by different mountains and we think that niche divergence could be the responsible for speciation in *Otatea* species. In *Otatea*, the morphology data set was more useful for studying recently diverged taxa than the molecular data set. It was advantageous to gather morphological, molecular and ecological data simultaneously in order to detect recently diverged taxa, because the molecular data alone was unable to distinguish recently formed species.

Furthermore, the species will be described based on the vegetative characters that supported the new taxa. Niche modeling corroborated that the groups recognized in the morphological analyses occupy different habitats. The molecular statistical parsimony network indicated that the ancestral genotype in *Otatea* is found in the population from Colombia. Kelchner and Clark (1997) in their study of *Chusquea* indicated this genus originated

in South America, migrating to Central and to North America. It could be possible that the same biogeographical pattern occurred in *Otatea* species.

We conclude that the two subspecies in *Otatea acuminata* can not be segregated and raised to the level of species. In addition we resolved that the population in Colombia indeed forms part of *O. fimbriata*. Therefore, based in the morphology tree- and character-based analyzes and corroborated by the modeling of ecological niche we decided to recognize seven entities in *Otatea*.

Further research considering additional DNA markers (AFLP, microsatellites, other nuclear DNA sequences) will allow to date time of origin of *Otatea* species. Timing will detect if Pleistocene climate fluctuations could be responsible for the process of speciation in *Otatea*. They will also allow to elucidate if some of the species had an hybrid origin.

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Appendix 1. Morphological characters

1.1. Culms

1. Habit: 0 = erect; 1 = apically arching/pendulous.
2. Culm internodes: 0 = all solid (at least when young); 1 = all hollow; 2 = some proximal internodes (including the basalmost ones) solid, distal internodes hollow.
3. Wall thickness (ratio of 2× wall thickness: culm diameter): 0 = walls very thin (ratio up to 0.15); 1 = walls thin (ratio 0.16–0.30); state 2 = walls moderately thick (ratio 0.31–0.45); 3 = walls thick (ratio 0.46–0.60); 4 = walls very thick (ratio 0.61–0.99).
4. Lacuna size: 0 = lacuna large, >1/3 the diameter of the culm; 1 = lacuna small, <1/3 the diameter of the culm.
5. Nodal line position: 0 = horizontal; 1 = dipping slightly below the bud.

1.2. Buds and branching

6. Branching pattern: 0 = intravaginal; 1 = extravaginal.
7. Branch complement (Londoño and Clark, 2002): 0 = 1 divergent branch; 1 = 1 or 2 divergent branches; 2 = 3 subequal, ascending branches.

1.3. Culm leaves

8. Abaxial sheath surface: 0 = stiff, dark, irritating hairs present; 1 = glabrous, no hairs present.
9. Relative size of the culm leaf with regard to sheat: 0 = same size; 1 = a half of the sheat; 2 = a third of the sheath.
10. Relative size of the culm leaf with regard to internode: 0 = same size; 1 = larger than internode; 2 = a half of the sheat; 3 = a third of the sheat.
11. Sheath apex: 0 = more or less horizontal; 1 = symmetrically convex; 2 = symmetrically concave.
12. Sheath apex (or summit or shoulders) indument: 0 = glabrous; 1 = ciliate; 2 = fimbriate.
13. Sheath summit extension: 0 = absent; 1 = present on one or both sides.
14. Oral setae: 0 = absent; 1 = present, whether adnate to the inner ligule or not.
15. Oral setae indument: 0 = glabrous; 1 = all long setae scabrous; 2 = only at base scabrous.
16. Oral setae color when live: 0 = brown; 1 = green; 2 = white; 3 = yellow; 4 = purple.
17. Oral setae length in mm: 0 = more than 13 mm; 1 = 4.5–12 mm.
18. Culm leaf blade position: 0 = erect to slightly spreading; 1 = reflexed.
19. Culm leaf blade shape: 0 = broadly triangular; 1 = more or less narrowly triangular.
20. Culm leaf blade midrib abaxial development: 0 = indistinguishable; 1 = visible or even prominent toward the apex.
21. Culm leaf sheath base indument: 0 = glabrous; 1 = ciliate; 2 = fimbriate

1.4. Foliage leaves

22. Sheath summit extension: 0 = absent; 1 = present on one or both sides.
23. Sheath summit indument: 0 = glabrous; 1 = fimbriate.
24. Sheath: 0 = rounded on the back; 1 = strongly keeled at least near the summit.
25. Sheath indument: 0 = glabrous; 1 = hispidous.
26. Outer ligule (contraligule): 0 = absent; 1 = present continuously along the width of the sheath summit; 2 = bilobed.
27. Outer ligule (contraligule), maximum length in mm of the lobes: 0 = 5 mm; 1 = 1 mm.
28. Oral setae: 0 = absent; 1 = present whether adnate to the inner ligule or not.
29. Oral setae connation: 0 = free; 1 = connate at base; 2 = connate 1/3 or more.
30. Oral setae consistency: 0 = coriaceous; 1 = papyraceous.
31. Oral setae color when live; 0 = purple; 1 = yellow; 2 = white; 3 = brown; 4 = green.
32. Oral setae indument: 0 = glabrous; 1 = all long setae scabrous.
33. Oral setae length in mm: 0 = more than 10 mm; 1 = 6 mm.
34. Fimbria growth: 0 = curly; 1 = straight.
35. Foliage leaf blade wide: 0 = less than 1.5 cm; 1 = more than 1.6 cm.
36. Midrib placement: 0 = centric; 1 = excentric (wider side of blade 1.3 times or more as wide as the narrower side).
37. Patch of cilia on the base of the blade abaxial side: 0 = absent; 1 = present.

38. Patch of cilia on the base of the blade abaxial side position: 0 = at one side of the central nerve; 1 = on both sides of the central nerve.
39. Patch of cilia on the base of the abaxial side density: 0 = very dense; 1 = dispersed.
40. Patch of cilia on the base of the abaxial side color: 0 = brown; 1 = yellow; 2 = white.

1.5. Synflorescences and spikelets

41. Maximum synflorescence length in cm: 0 = 9; 1 = 10; 2 = 12; 3 = 15.
42. Maximum number of spikelets: 0 = 7; 1 = 8; 2 = 15; 3 = 30.
43. Spikelets length in cm: 0 = 2; 1 = 3; 2 = 3.5; 3 = 4.
44. Rachilla joint length in mm: 0 = 4; 1 = 5; 2 = 6; 3 = 7.
45. Glume abaxial surface: 0 = scabrous; 1 = glabrous.
46. Lemma abaxial surface: 0 = pubescent; 1 = scabrous; 2 = scabrid; 3 = glabrous.

1.6. Foliar micromorphology

47. Papillae on the long cells in the stomatal zone (abaxial): 0 = absent; 1 = present.
48. Papillae on the long cells in the interstomatal zone (abaxial): 0 = absent; 1 = present.
49. Papillae on the adaxial surface position: 0 = present on the long cells only; 1 = present on both bulliform and long cells.
50. Distribution of stomates on foliage leaf blades: 0 = present and common on the abaxial surface only; 1 = present and common on both surfaces.
51. Vertically tall and narrow silica bodies (abaxial, intercostal): 0 = present; 1 = absent.
52. Vertically tall and narrow silica bodies (abaxial, costal): 0 = present; 1 = absent.
53. Saddle-shaped silica bodies (abaxial, costal): 0 = present; 1 = absent.
54. Horizontal dumbbell-shaped silica bodies (abaxial, costal): 0 = present; 1 = absent.

Appendix 2. Morphological character matrix

Inapplicable data = "–", not seen = "?", A = 0–1

	000000000111111111122222222223333333333444444444455555
	123456789012345678901234567890123456789012345678901234
Olrecta	11100000120201030010001100-100100000101-21341300001011
Oa4	11300020100200---010101000-0-----0001112??????00100101
Oa6	11300020100200---010101000-0-----0001112??????00110100
Oa10	1231002010020100-000101000-0-----00011121222000100101
Oa9	1231002010020100-000101000-0-----00011121222000100100
Oa17	10--0021101110---010111000-0-----0000---12220000110101
Oa2	10--0020200200---000101000-0-----0000---12220011100110
Oa1	10--0020200200---000101000-0-----0000---??????11100110
Oa3	00--0021221200---000001000-0-----0001112??????00100101
Oa16	00--0021221200---000001000-0-----0000---??????00100100
Of26	0120102001020100001010110 1-1103000111000330012000001 00
Of23	0120102001020100001010110 1-1103000111000330012000001 01
Of24	0120102001020100001010110 1-1103000111000330012000001 00
Of28	1120102001020100 00A0101101-1103000111000?????00000110
Oa25	00--0021121200---000101000-0-----0001112??????11100101
Oa20	00--0020200200---000101000-0-----0001112??????11100101
Oa18	00--0020200200---000101000-0-----0001012??????11100101
Of5	0241001111211102001100011211012 00-111102??????11100000
Of5(a)	0241001111211102001100011211012 00-111102??????11000000
Of21	0241002122221114111001000 0-100001-011102??????00000101
Og27	1110002020220111011000100 0-1004110011112003110000101 1
Ospnov22	1140002001221111011121100 0-1004001001012??????00101011
Oa19	12310021200200---000001000-0-----0010---??????11100101
Oa12	1231002010020120000010101 0-0-----00011121222001110010 0
Oa18	1231002010020120000010100 0-0-----00011121222000010010 0
Of15	0220011011200101011000000 20120000-111101??????00100001
Of11	0220011011200101011000000 20120000-111101??????00100001
Of7	0220011011200101011000000 20120000-111101??????00100001
Oa14	10--0020100201200000101010-1001010011112122200110010 1
Oa13	10--0020100201200000101010-1001010011112122200110010 1

Appendix 3. Vouchers and specimens examined

Olmeca recta Soderstr. MEXICO, Veracruz: E. Ruiz-Sanchez 132 (XAL).

Otatea acuminata (Munro) C. Calderón and Soderstr. MEXICO, Colima: E. Ruiz-Sanchez et al. 101, 176 (XAL). Chiapas: E. Ruiz-Sanchez and J.L. Martínez 119 (XAL). Durango: E. Ruiz-Sanchez and P. Carrillo-Reyes 113 (XAL). Guanajuato: E. Ruiz-Sanchez and F. Rodriguez-Gomez 173 (XAL). Guerrero: P. Carrillo-Reyes 4863 (XAL). Hidalgo: E. Ruiz-Sanchez and P. Carrillo-Reyes 114 (XAL). Jalisco: E. Ruiz-Sanchez et al. 97, 102 (XAL). Michoacán: E. Ruiz-Sanchez et al. 181, 182 (XAL). Nayarit: E. Ruiz-Sanchez et al. 96 (XAL). Oaxaca: E. Ruiz-Sanchez and J.L. Martínez 125 (XAL). Puebla: E. Ruiz-Sanchez and J.L. Martínez 126 (XAL). Sinaloa: E. Ruiz-Sanchez et al. 105 (XAL). Sonora: E. Ruiz-Sanchez et al. 112 (XAL). Veracruz: E. Ruiz-Sanchez and F. Rodriguez-Gomez 103 (XAL).

Otatea fimbriata Soderstr. COLOMBIA, Norte de Santander: X. Londoño and E. Ruiz-Sanchez 987 (CUCV). MEXICO, Colima: E. Ruiz-Sanchez et al. 183 (XAL). Chiapas: E. Ruiz-Sanchez and J.L. Martínez 118 (XAL); E. Ruiz-Sanchez 136, 155 (XAL). Estado de Mexico: E. Ruiz-Sanchez et al. 179 (XAL). Jalisco: E. Ruiz-Sanchez et al. 130, 186, 189 (XAL). Oaxaca: P. Carrillo-Reyes 4986 (XAL); E. Ruiz-Sanchez et al. 217 (XAL).

Otatea glauca L.G. Clark and G. Cortés MEXICO, Chiapas: E. Ruiz-Sanchez 144 (XAL).

Otatea sp. nov. *Chiapas* MEXICO, Chiapas: P. Carrillo-Reyes 5144 (XAL); E. Ruiz-Sanchez 147 (XAL).

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