



Parametric and non-parametric species delimitation methods result in the recognition of two new Neotropical woody bamboo species [☆]



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ABSTRACT

The Neotropical woody bamboo genus *Otatea* is one of five genera in the subtribe Guaduinae. Of the eight described *Otatea* species, seven are endemic to Mexico and one is also distributed in Central and South America. *Otatea acuminata* has the widest geographical distribution of the eight species, and two of its recently collected populations do not match the known species morphologically. Parametric and non-parametric methods were used to delimit the species in *Otatea* using five chloroplast markers, one nuclear marker, and morphological characters. The parametric coalescent method and the non-parametric analysis supported the recognition of two distinct evolutionary lineages. Molecular clock estimates were used to estimate divergence times in *Otatea*. The results for divergence time in *Otatea* estimated the origin of the speciation events from the Late Miocene to Late Pleistocene. The species delimitation analyses (parametric and non-parametric) identified that the two populations of *O. acuminata* from Chiapas and Hidalgo are from two separate evolutionary lineages and these new species have morphological characters that separate them from *O. acuminata* s.s. The geological activity of the Trans-Mexican Volcanic Belt and the Isthmus of Tehuantepec may have isolated populations and limited the gene flow between *Otatea* species, driving speciation. Based on the results found here, I describe *Otatea rzedowskiorum* and *Otatea victoriae* as two new species, morphologically different from *O. acuminata*.

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1. Introduction

The Neotropical woody bamboo clade, one of the four main lineages of bamboos (Bambusoideae) comprises three monophyletic subtribes (Arthrotyliidiinae, Chusqueinae and Guaduinae) with a total of 377 bamboo species described as endemic to the Americas (Ruiz-Sanchez et al., 2008, 2011a; Fisher et al., 2009; Tyrrell et al., 2012; BPG, 2012; Kelchner and BPG, 2013; Clark et al., 2015). Recent phylogenetic analyses using chloroplast markers (Sungkaew et al., 2009; Kelchner and BPG, 2013) and full plastomes (Wysocki et al., 2015) found a sister relationship between the Neotropical woody bamboo clade and the Paleotropical woody bamboo clade. These two clades form the tribe Bambuseae, which is sister to the Olyreae tribe (herbaceous bamboos), and these two tribes are sister to the Temperate woody bamboo tribe, Arundinarieae, making woody bamboos paraphyletic with respect to Olyreae; however Kelchner and BPG (2013) could not reject the monophyly of woody bamboos based on their result using the Shimodaira–Hasegawa test. Alternatively, using three nuclear loci

Triplett et al. (2014) found monophyly for woody bamboos sister to Olyreae and a complex reticulate evolutionary history of woody bamboos. Recent phylogenetic analyses suggest that hybridization has played an important role in bamboo diversification (Triplett et al., 2010; Zhang et al., 2012; Yang et al., 2013). Triplett et al. (2014) confirmed that hybridization has been recurrent in bamboo evolution, generating allohexaploid species in paleotropical and intergeneric hybrids among the temperate woody bamboos. However, to the date there is no molecular evidence to confirm or reject the potential hybrid origin of Neotropical woody bamboo genera and species.

The most recent molecular phylogenetic studies found a sister relationship between Arthrotyliidiinae and Guaduinae (Ruiz-Sanchez et al., 2011a; Tyrrell et al., 2012), with these two subtribes sister to Chusqueinae (Fisher et al., 2009; Kelchner and BPG, 2013). Guaduinae is a monophyletic subtribe, supported by molecular characters (Ruiz-Sanchez et al., 2008, 2011a; Tyrrell et al., 2012). Its morphological character combination includes usually abundant stomata on the adaxial epidermis and well developed papillae (Ruiz-Sanchez et al., 2008; BPG, 2012), which are diagnostic for the subtribe.

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The most recent update of the tribe and subtribe classification of the bamboos published by the Clark et al. (2015) indicates that Guaduinae comprises 50 described species in five genera: *Apoclada* McClure with one species, *Eremocaulon* Soderstr. & Londoño with four species, *Guadua* Kunth with 32 species, *Olmecca* Soderstr., with five species, and *Otatea* (McClure & E. W. Sm.) Calderón & Soderstr., with eight species. The Guaduinae subtribe is distributed from Mexico to Uruguay and Argentina (Judziewicz et al., 1999; Ruiz-Sanchez et al., 2008; Ruiz-Sanchez et al., 2011a, 2011b). Mexico has the second greatest diversity of Guaduinae species, with 20 described species in three genera (*Guadua*, *Olmecca* and *Otatea*) followed by Brazil with the same number of genera (*Apoclada*, *Eremocaulon* and *Guadua*), but with more *Guadua* species (Londoño and Ruiz-Sanchez, 2014).

Otatea has eight described species, all of which are present in Mexico, seven of which are endemic, and one (*Otatea fimbriata* Soderstr.) that is also distributed in Central America and Colombia (Clark and Cortés, 2004; Ruiz-Sanchez and Sosa, 2010; Ruiz-Sanchez et al., 2011b; Ruiz-Sanchez, 2012, 2013). *Otatea* species grow on slopes in tropical dry forests, xerophilous scrub, in the ecotone between oak forest and tropical dry forest, humid pine-oak forests and cloud forests, at elevations of 150–2100 m a.s.l. (Ruiz-Sanchez et al., 2011b; Ruiz-Sanchez, 2012). *Otatea acuminata* has the widest geographical distribution in Mexico (Fig. 1), mainly occurring on the slopes of tropical dry forest along the Pacific coast and Trans-Mexican Volcanic Belt. Previous phylogenetic analyses revealed *Otatea* to be a monophyletic genus, sister to *Olmecca* (Ruiz-Sanchez et al., 2008, 2011a; Ruiz-Sanchez and Sosa, 2010, 2015; Tyrrell et al., 2012) and supported by two morphological synapomorphies: three subequal ascending branches per node and pubescent lemmas (Ruiz-Sanchez et al., 2008).

Ruiz-Sanchez and Sosa (2010) applied the Wiens and Penkrot (2002) method for delimiting species to *Otatea* using molecular, morphological and ecological data. They were able to delimit seven species, but *O. acuminata* was polyphyletic. Four of the seven

species were new species, later described by Ruiz-Sanchez et al. (2011b). Ten years ago, Sites and Marshall (2003, 2004) identified several methods for delimiting species and called them operational criteria for delimiting species boundaries. All these methods for delimiting species are now known as *non-parametric analyses* (Carstens et al., 2013). In recent years two parametric (BP&P = Bayesian Phylogenetics and Phylogeography and spedeSTEM = Species Tree Estimation Using Maximum Likelihood) coalescent-based methods for delimiting species have been developed (Yang and Rannala, 2010; Ence and Carstens, 2011), along with other coalescent-based methods (reviewed in Fujita et al., 2012). BP&P (Bayesian Phylogenetics and Phylogeography) is a coalescent-based method that uses reversible-jump MCMC, which identifies evolutionary lineages given multilocus data and a starting tree (Yang and Rannala, 2010).

Recent field work and a review of herbarium specimens indicate that some of the morphological characters (persistent culm leaf blades, culm leaf and foliage leaf size among others) of two *O. acuminata* populations are not consistent with the type of the species, and previous molecular analysis using chloroplast (cpDNA) and nuclear markers (nrDNA) indicates that this species is polyphyletic and is an excellent candidate for species delimitation analysis and taxonomic revision (Ruiz-Sanchez and Sosa, 2010; Ruiz-Sanchez et al., 2011a). Hence, I applied one parametric (BP&P) coalescent-based method for delimiting species using chloroplast and nuclear sequence data and one non-parametric tree-based method using both (cpDNA + nrDNA) sequence data and morphological characters. I followed the general lineage concept of species as postulated by de Queiroz (1998), which states that species are *segments of population level evolutionary lineages*. The main goals of this study were: (1) to delimit species in the genus *Otatea* using molecular and morphological data applying parametric and non-parametric methods; (2) to infer whether there is more than one species in *O. acuminata*, and (3) to determine the time of divergence of *Otatea*.

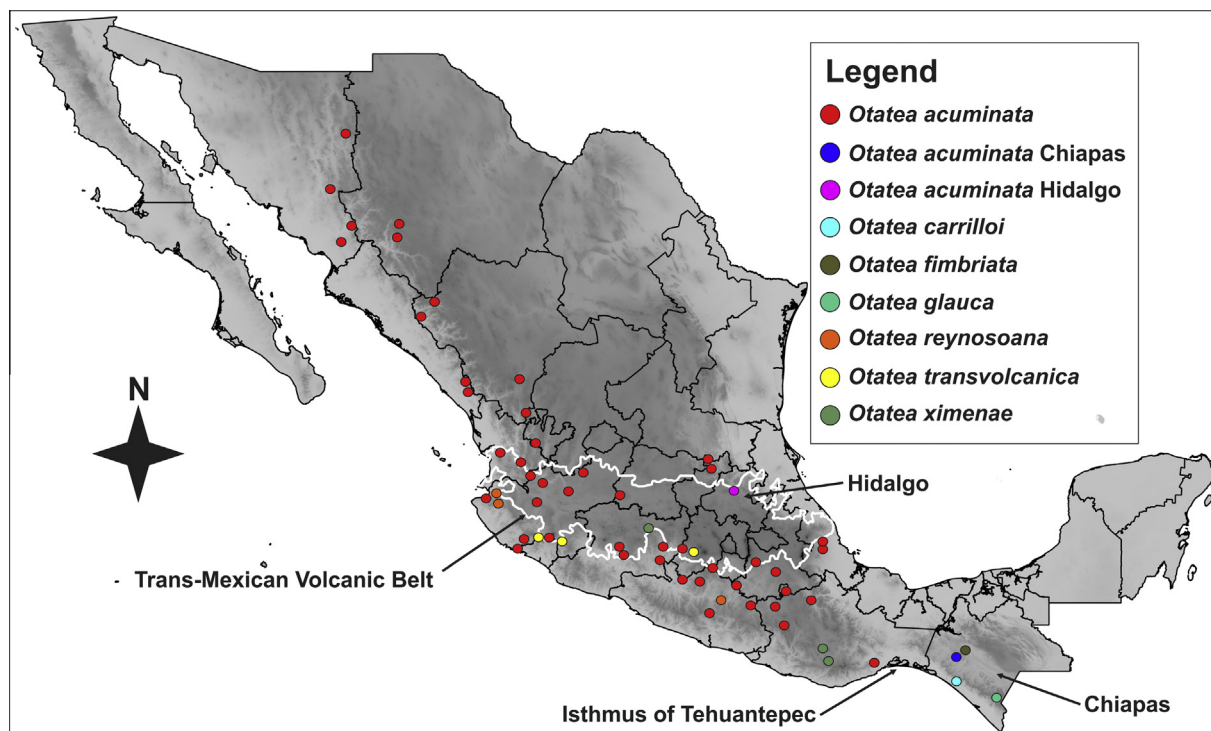


Fig. 1. Geographical distribution of *Otatea* species in Mexico.

2. Materials and methods

2.1. Taxon sampling

A total of 22 accessions were included in the analyses, 21 as the ingroup and one as the outgroup based on the results of Ruiz-Sanchez and Sosa (2010) and Ruiz-Sanchez et al. (2011a). For the ingroup seven of the eight *Otatea* species were included as follows: four *O. acuminata* specimens, three each of *Otatea carrilloi*, *O. fimbriata*, *Otatea reynosoana*, *Otatea transvolcanica* and *Otatea ximena* and two *Otatea glauca* specimens. Their geographical distributions are shown in Fig. 1. For the outgroup *Olmea recta* was used (see Supplementary Material 1).

2.2. DNA matrices

Sequences of five chloroplast (cpDNA = *atpF-atpH*, *matK*, *psbK-psbI*, *rbcL* and *rpl32-trnL*) and one nuclear (nrDNA = ITS) markers were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>); the accession numbers are given in Supplementary Material 1.

2.3. Morphological matrix

The morphological matrix of 47 characters was constructed with WinClada (Nixon, 2002) (Supplementary Material 2). Character selection was based on the list and illustrations by L.G. Clark in “Bamboo Biodiversity” (<http://www.eob.iastate.edu/research/bamboo>) and Ruiz-Sanchez and Sosa (2010). Characters were scored by examining live material and herbarium specimens. The vouchers and specimens examined are also listed in Supplementary Material 3.

2.4. Phylogenetic analyses

Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were conducted using Garli v.2.0 (<http://garli.googlecode.com>) and MrBayes v.3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), respectively. BI and ML analyses were performed on the concatenated molecular data.

First, jModelTest v.2.1.6 (Darriba et al., 2012) was used to detect the model of molecular evolution that best fit our individual (cpDNA and nrDNA) molecular data sets using the AICc (Akaike Information Criterion).

The ML analyses of the concatenated molecular (cpDNA + nrDNA) data matrix were performed using Garli v.2.0 with 1000 non-parametric bootstrap replicates run on the Garli web service (http://www.molecularrevolution.org/software/phylogenetics/garli/garli_create_job), and nodes were considered highly supported when bootstrap values were $\geq 85\%$. The BI analyses of the concatenated molecular data matrix and of the molecular and morphological data matrix (the model used for morphology was rates = gamma, as per the MrBayes manual) were performed using MrBayes v.3.2.2. Two independent runs were done for each data matrix. For each run I employed one cold and three heated chains, set to run for 10,000,000 generations, sampling one tree every 1000 generations. Stationarity was determined by the likelihood scores for time to convergence, and sample points collected prior to stationarity were eliminated (25%). Posterior probabilities (PP) for supported clades were determined by a 50% majority-rule consensus of the trees retained after burn-in. I considered those nodes with PP ≥ 95 to be strongly supported. BI analyses were run on CIPRES Science Gateway (Miller et al., 2010).

2.5. Species delimitation

I used two methods to delimit the species in *Otatea*. The first is the non-parametric method using the morphology and distribution data within a phylogenetic framework based on the molecular data. The second is the BP&P parametric analysis. I used the program BP&P v.2.0 that incorporates a Bayesian modeling approach to generate speciation probabilities for closely related taxa from multilocus data (Rannala and Yang, 2003; Yang and Rannala, 2010). The program takes into account gene tree uncertainty and lineage sorting and assumes no gene flow among species after divergence (Yang and Rannala, 2010). I ran the analyses for 400,000 generations, sampling every 2 generations and specified a burn-in for the first 20,000 generations. I performed preliminary analyses using algorithms 0 and 1 with different fine-tuning parameters and using the total evidence (molecular + morphology) tree topology to specify the guide tree. Because the BP&P program has been shown to be sensitive to the choice of prior distributions of the theta and tau (Zhang et al., 2011), I followed the approach of Leaché and Fujita (2010) and performed the analyses using three combinations of priors that represent different population sizes and different ages for the root in the species tree. The first combination of priors assumed a large population size and deep divergence: $\theta G(1, 10)$ and $\tau_0 G(1, 10)$. The second combination of priors assumed small ancestral population sizes and shallow divergence among species $\theta G(2, 2000)$ and $\tau_0 G(2, 2000)$. The third combination assumed a mixture of priors with large population sizes and shallow divergence among species $\theta G(1, 10)$ and $\tau_0 G(2, 2000)$. BP&P then generates an *a posteriori* distribution of speciation models containing different numbers of species. Speciation probabilities are estimated from the sum of probabilities of all models for speciation events at each node in the guide tree. Following Smith et al. (2013), for all three prior scenarios I considered all nodes that had speciation probabilities >0.95 to be species.

2.6. Molecular dating

To estimate the divergence times of *Otatea*, I used a different matrix. Representative species from the three known Bambusoideae tribes (Bambuseae, Arundinarieae and Olyreae) and *Alopecurus pratensis* (Pooideae) were used. Chloroplast *matK* and nuclear ITS markers were downloaded from GenBank (Supplementary Material 1). I implemented a Bayesian approach using BEAST v.1.8 (Drummond and Rambaut, 2007) to estimate divergence times. The HKY + G + I model of molecular evolution was used based on the results of the AICc analysis, under an uncorrelated lognormal relaxed clock model. Based on the results of Sungkaew et al. (2009), Bouchenak-Khelladi et al. (2008) and Ruiz-Sanchez (2011), four nodes were constrained: Bambusoideae, Arundinarieae, Bambuseae and Olyreae. The Yule speciation process was used to model the tree prior. Five secondary points derived from Bouchenak-Khelladi et al. (2010), Ruiz-Sanchez (2011) and Prasad et al. (2011) were used with a normal distribution. For the root I used a mean age of 49 Ma, SD = 7 (60.5–37.4 Ma), for the Bambusoideae stem node a mean of 29 Ma, SD = 9 (43.8–14.2 Ma), for the Arundinarieae crown node a mean of 10.6 Ma, SD = 5 (18.8–2.3 Ma), for the Bambuseae crown node a mean of 26.7 SD = 4 (33.9–19.5 Ma), and for the Olyreae crown node a mean of 25 Ma, SD = 4.5 (32.4–17.6 Ma). Two independent 40^7 generation runs were performed with a random starting tree, sampling every 1000 generations. Tracer 1.5 (<http://tree.bio.ed.ac.uk/software/tracer/>) was used to assess convergence and estimate effective sample sizes (ESS) for all parameters. The results were summarized in a single tree using LogCombiner and Tree Annotator v.1.8 (Drummond and Rambaut, 2007) and visualized with FigTree v.1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

3. Results

The total number of aligned base pairs (bp) for each cpDNA and nrDNA marker was: *atpF–atpH* = 621; *matK* = 849; *psbK–psbI* = 422; *rbcl* = 623 and *rpl32–trnL* = 846; ITS = 606. The molecular evolution models produced by the jModelTest analyses are: *atpF–atpH* = HKY + I; *matK* = TrN; *psbK–psbI* = F81; *rbcl* = F81 and *rpl32–trnL* = F81 and ITS = HKY + I + G, respectively.

The aligned cpDNA matrix was 3365 bp long, of which 29 bp were variable polymorphic sites. The aligned nrDNA matrix was 606 bp long, of which 64 bp were variable polymorphic sites. The concatenated cpDNA + nrDNA aligned matrix was 3971 bp long. The morphological matrix had 47 characters with 5 uninformative characters and 42 that were parsimony informative (Supplementary Material 4). The concatenated cpDNA + nrDNA + morphology matrix was 4018 sites long.

3.1. Phylogenetic analyses

Two sets of phylogenetic analyses were conducted: the concatenated molecular data using ML and BI, and the concatenated molecular and morphological data using BI. The partitioned molecular (cpDNA + nrDNA) analysis using ML and BI retrieved similar topologies. The parametric bootstrap support and posterior probabilities for those analyses are written below the nodes and are shown in Fig. 2a. The partitioned molecular (cpDNA + nrDNA) and morphological analysis using BI is shown in Fig. 2a. Two main clades were identified. The first clade comprised *O. acuminata* Chiapas, *O. fimbriata*, *O. ximena*. These species have geographical distribution in the states of Chiapas for *O. acuminata*, *O. fimbriata*,

and Michoacán and Oaxaca for *O. ximena* (Fig. 1). *O. fimbriata*, *O. ximena* and *O. acuminata* Chiapas are well supported (PP = 0.99–1.0 posterior probabilities) as reciprocally monophyletic lineages. *O. fimbriata* is sister to *O. ximena* (PP = 0.99) and *O. acuminata* Chiapas is sister to both these species (PP = 1.0). The second clade comprises the following species: *O. carrilloi*, *O. glauca*, *O. acuminata*, *O. acuminata* Hidalgo, *O. reynosoana* and *O. transvolcanica* (Fig. 2a). All the species formed monophyletic lineages, however the position of *O. carrilloi* is not resolved though it is well supported (PP = 1.0), as is that of *O. glauca* (PP = 1.0); both species are endemic to Chiapas (Fig. 1). *O. reynosoana* is distributed in the states of Guerrero and Jalisco, and *O. transvolcanica* in the states of Colima, Jalisco and Mexico. Finally, *O. acuminata* is widely distributed throughout Mexico (Fig. 1). *O. reynosoana* is sister to *O. transvolcanica* but with low support (PP = 0.78); *O. acuminata* is sister to these two species (PP = 0.99), and *O. acuminata* Hidalgo is sister to all of them (PP = 1.0). The main result is that *O. acuminata* is not monophyletic with two populations from Chiapas and Hidalgo separated into different lineages.

3.2. Species delimitation

The non-parametric method identified nine evolutionary lineages: *O. carrilloi*, *O. fimbriata*, *O. glauca*, *O. reynosoana*, *O. transvolcanica* and *O. ximena*, *O. acuminata* s.s., *O. acuminata* Chiapas and *O. acuminata* Hidalgo (Figs. 1 and 2a).

The parametric Bayesian species delimitation result for *Otatea* using BP&P analysis and the guide tree is shown in Fig. 2b. I obtained qualitatively similar results using the different algorithms and starting trees. Assuming nine species, the Bayesian

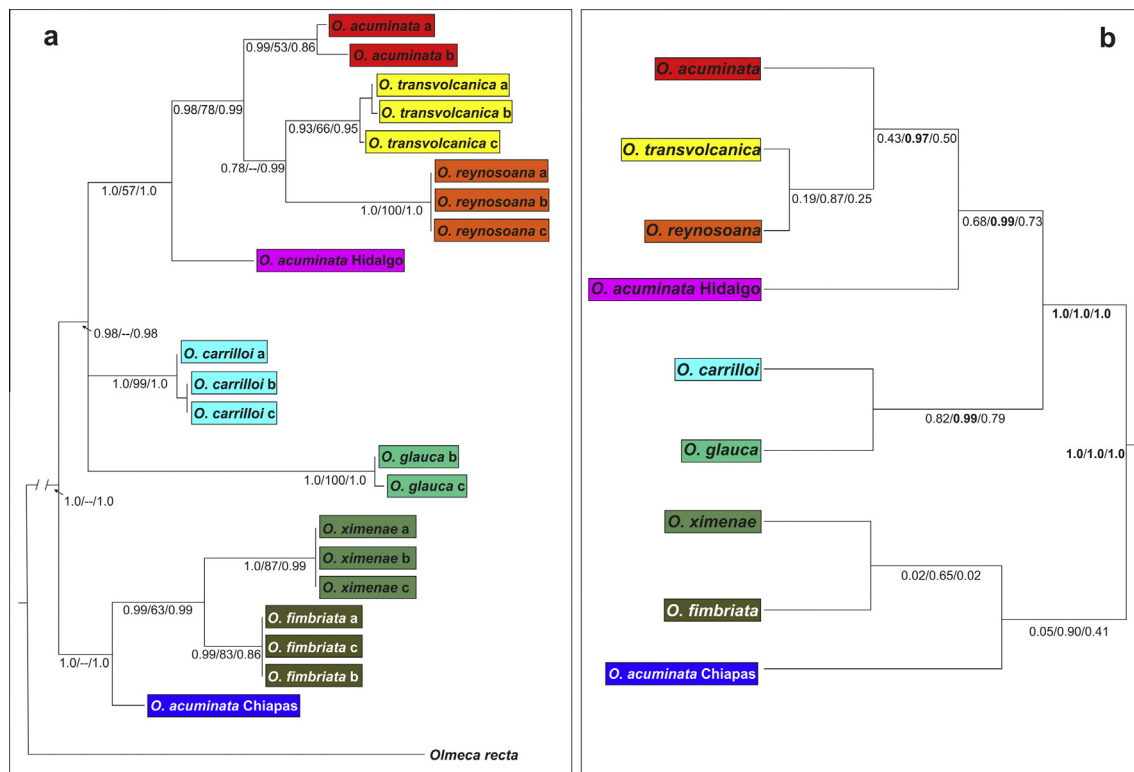


Fig. 2. (a) Bayesian 50% majority consensus tree based on the partitioned chloroplast (*atpF–atpH*, *matK*, *psbK–psbI*, *rbcl* and *rpl32–trnL*) and nuclear (ITS) gene markers, and morphology, showing the relationships among *Otatea* species in Mexico. Numbers below the branches at left are posterior probabilities from the partitioned regions (cpDNA + nrDNA + morphology), numbers in the center are non-parametric ML bootstrap support and numbers to the right are posterior probabilities of the BI using cpDNA and nrDNA. (b) Bayesian species delimitation results for *Otatea* species assuming a 9-species guide tree. Speciation probabilities are provided for each node under a combination of priors for θ and τ_0 : left θ G(1, 10) and τ_0 G(1,10) (large population sizes and deep divergences), central θ G(2, 2000) and τ_0 G(2, 2000) (small population sizes and shallow divergences) and right θ G(1, 10) and τ_0 G(2, 2000) (large population sizes and shallow divergences).

species delimitation supports the guide tree with speciation probabilities from 0.02 to 1.0 at the nodes. The highest probability delimitations was obtained for the second combination of priors that assumed small ancestral population sizes and shallow divergences among species: θ $G(2, 2000)$ and τ_0 $G(2, 2000)$ (Fig. 2b). Four species are supported by speciation probabilities >0.95 on the species guide tree; these species are *O. carrilloi*, *O. glauca*, *O. acuminata* and *O. acuminata* Hidalgo, while the rest of the species have speciation probabilities <0.95 (Fig. 2b). Speciation probability for the clade of *O. acuminata* Chiapas, *O. fimbriata* and *O. ximena* is 1.0 (Fig. 2b).

3.3. Divergence time estimates

The BEAST analyses suggest that the split between the *Otatea* clade and the other Guaduininae genera (*Olmeca* and *Guadua*) was 15.7 Ma (95% HPD 23–8.75 Ma; Fig. 3). The divergence of the *Otatea* clade likely began 6.11 Ma (95% HPD 10.15–2.73 Ma; Fig. 3). The speciation events occurred from the Late Miocene to the Late Pleistocene (Fig. 3).

3.4. Morphological species description

Based on the parametric and non-parametric results reported above, here I describe two new species of *Otatea*:

3.4.1. *Otatea rzedowskiorum* Ruiz-Sanchez, sp. nov., Figs. 4–6

TYPE:— MEXICO. Chiapas: Chiapa de Corzo, highway from Tuxtla Gutierrez to San Cristobal de Las Casas, km 14.5, El Federalista Bridge, 16°42'45" N, 2°54'40.3"W, 1017 m a.s.l., 20 June 2013, tropical deciduous forest, M T. Mejía-Saulés & E. Ruiz-Sanchez 2192 (holotype: IEB!; isotypes: HEM!, ISC!, MEXU!, XAL!).

O. rzedowskiorum is similar to *Otatea ramirezii* and *Otatea victoriana*, but differs in having three subequal branches per node, develops auricle-like structures on both sides of the culm sheath, has

wider foliage leaf blades, develops fewer spikelets than *O. ramirezii* and spikelets are smaller with glabrous glumes.

Rhizome necks 3.5–10 cm long. Culms 1.5–4.5 m tall, 0.8–1.5 cm in basal diameter, erect to slightly apically arching; internodes 10–17.5 cm long, terete, glabrous, green and pruinose when young, solid, becoming fistulose with age. Culm leaves 11–19.5 cm long, rectangular, non-overlapping, deciduous; sheaths 8–12 cm long, 3–5 cm wide at the base, rectangular, glabrous, margins ciliate, leaf blades 2.5–7.5 cm long, triangular, erect, persistent, shorter than the sheaths, glabrous, margins ciliate, apex acuminate; abaxially the leaf blades and the sheaths form a continuous surface without division both are fused, adaxially the leaf blades and the summit of the sheaths form a sinuate division, inner ligule a coriaceous rim 0.1–0.7 mm long, sinuate and ciliate; oral setae absent; fimbriae borne in two irregular auricle-like structures at the summit of the sheath on both sides, 3–5 mm long, ca. 0.05 mm wide, terete, strongly fused at the base becoming deciduous when old, curly. Branching intravaginal; three main branches per node, subequal, sometimes the basal node develops only one branch and these diverge from each other and rebranch, 40–70 (110) cm long, diverging from the main culm at 45–60°, and rebranching in second and third order; supranodal ridge pronounced; nodal line horizontal. Foliage leaves 2–3 per complement; sheaths glabrous, rounded on the back; oral setae absent; fimbriate at sheath shoulders, the fimbriae 1–2 mm long, ca. 0.05 mm in diameter, terete, deciduous, erect, glabrous; outer ligule an irregular glabrous rim up to 0.2 mm long; inner ligule 0.2–0.3 mm long, truncate, ciliate; pseudopetioles ca. 0.5 mm long; blades (6.5) 9–11 (11.5) cm long, 3–4 mm wide, linear to linear-lanceolate, adaxially green and glabrous, abaxially, with a patch of white cilia at the base extending along one side of the midrib for 2–6 mm, the base attenuate, the apex acute, the margins weakly serrulate. Synflorescences 4–7 cm long, paniculate, with 4–6 spikelets; pedicels 5–22 mm long, angular, glabrous to slightly scabrous. Spikelets 1.5–2.5 cm long, green, with 2–3 florets, upper floret sometimes sterile; rachilla joints 5.5–7 mm long, scabrous

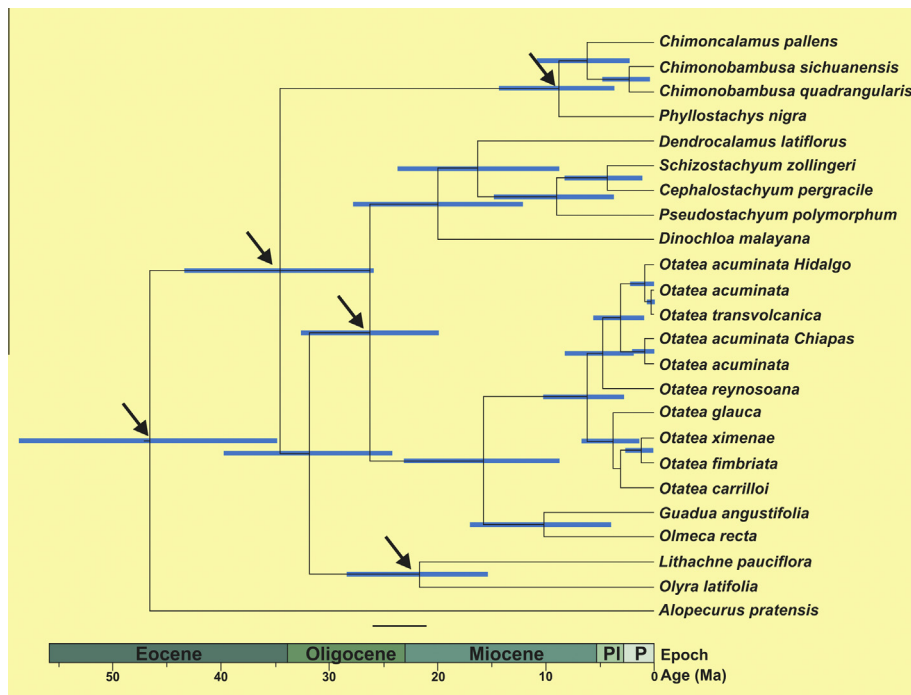


Fig. 3. Chronogram based on a Bayesian approach to the *Otatea* species in Mexico from partitioned chloroplast (*matK*) and nuclear ITS gene markers. Blue bars indicate 95% highest posterior density (HPD) intervals for node age estimates. Pl, Pliocene; P, Pleistocene; Ma, million years ago. Black arrows indicate calibration points.



Fig. 4. *Otatea rzedowskiorum*. A. Culm fragment showing culm leaves. B. Culm leaf adaxial view. C. Culm leaf abaxial view. D. Branch complement showing three subequal branches. E. Culm fragment, showing branches and foliage leaves. F. Ligular area of foliage leaf, showing fimbriae and patch of cilia at the base. Based on M.T. Mejía-Saulés & E. Ruiz-Sanchez 2192.

and pubescent at the apex of each joint; glume narrowly triangular and navicular, abaxially glabrous; glume I 5.5–6.5 mm long including the awn, 3-nerved, the awn 1–3.5 mm long; glume II 5.5–7.5 mm long including the awn, 3-nerved, the awn 2–3 mm long; lemmas 10–13 mm long including the awn, narrowly triangular and navicular, abaxially slightly scabrous 5-nerved, the awn 2–4 mm long, antrorsely scabrous; paleas 7.5–8.5 mm long, the keels glabrous to slightly scabrous, the sulcus scabrous-pubescent, wing glabrous to scabrous at the apex, apex bifid the teeth antrorsely scabrous. Lodicules 3, abaxially glabrous, margins ciliate, dark brown, the anterior pair 1–1.2 mm long, the posterior one 0.9–1 mm long; ovary 1–1.5 mm long, dark amber, glabrous. Caryopsis 7–8 mm long, sublinear, tapering to a narrow beak, dark amber.

3.4.2. Habitat and distribution

This species is only known from three populations in the state of Chiapas, Mexico two of which are in protected areas (Cascada el Aguacero and Cañón del Sumidero). *O. rzedowskiorum* inhabits tropical dry forest on calcareous soils from 600 to 1000 m a.s.l.

elevation, with a mean annual temperature of 26.7 °C, and annual precipitation of 973.6 mm.

3.4.3. Etymology

The specific epithet honors Dr. Jerzy Rzedowski and his wife Dr. Graciela Calderón de Rzedowski, in recognition of their achievements over an entire lifetime devoted to botany.

3.4.4. Phenology

The first record of flowering in this species is from 1984 and it was not recorded flowering again until last year (2014). This suggests a flowering cycle as long as 30 years for this species.

3.4.5. Additional specimens examined

MEXICO. Chiapas, El Aguacero, 610 m, 18 October 2013 A. Ortíz-Rodríguez & E. Ruiz-Sanchez 516 (IEB, XAL); Chiapas: El Aguacero, the Río La Venta Canyon, 540–660 m a.s.l., 19 November 1984 (fl), Davidse et al. 30076 (ISC, MO); on the Tuxtla to San Cristobal highway, El Federalista Bridge km 14.5, 1017 m a.s.l., 7 November 2005 E. Ruiz-Sanchez & J.L. Martínez 119 (XAL); on the Tuxtla to

San Cristobal highway, El Federalista Bridge km 14.5, 1017 m a.s.l., 26 September 2006 E. Ruiz-Sanchez & J. Pacheco 150 (XAL); In the Sumidero Canyon at La Atalaya Point, 10 January 2014 (fl), E. Ruiz-Sanchez, M.A. Perez Farrera & W. Wysocki 467 (IEB, XAL).

3.4.6. *O. victoriae* Ruiz-Sanchez sp. nov., Figs. 7 and 8

TYPE:— MEXICO. Hidalgo: Cardonal, at 6 km from the intersection with Dos Ríos on the way to Metznoxtla 20°37'39.3"N, 98°57'31.5"W, 1792 m a.s.l., 27 September 2014, xerophilous scrub, E. Ruiz-Sanchez, E. Gándara & I. Gúzman 484, (holotype: IEB!; isotypes: ISC!, MEXU!, XAL!).

O. victoriae is similar to *O. ramirezii* and *O. rzedowskiorum*, but differs in having three unequal branches per node and has smaller foliage leaf blades, lack of abaxial patch of white cilia but has scabrous fimbriae and scabrous sheaths.

Rhizomes with necks 10–30 cm long. Culms 2–5 m tall, 1–2 cm in basal diameter, erect; internodes 6.5–31 cm long, terete, glabrous, green and pruinose when young, solid or hollow, when hollow the walls 2–4 mm wide, the lacuna occupying more than half of the culm width. Culm leaves 7.5–20 cm long, rectangular, non-overlapping, deciduous; sheaths 6.5–13.8 cm long, 3.5–6 cm

wide at the base, rectangular, glabrous, margins minutely ciliate, leaf blades 0.8–7 cm long, triangular, erect, persistent and shorter than the sheaths, glabrous, margins minutely ciliate, apex acuminate; abaxially the leaf blades and the sheaths form a continuous surface without division, adaxially the leaf blades and the summit of the sheaths form a symmetrically convex division, inner ligule with a coriaceous rim 0.2–0.5 mm long, sinuate and minutely ciliate; oral setae absent; fimbriae at the summit of the sheath on both sides, 1.5–2 mm long, ca. 0.02 mm wide, terete, scabrous, deciduous, curly. Branching intravaginal; three main branches per node, the central borne first and it is twice the size of the two laterals and has rebranching, 10–100 cm long, diverging from the main culm at 45°, and rebranching in second and third order; supranodal ridge pronounced; nodal line horizontal. Foliage leaves 3–5 per complement; sheaths scabrous, rounded on the back; oral setae absent; fimbriae at the summit of the sheath on both sides of the blade, 0.5–1 mm long, ca. 0.01 mm wide, terete, scabrous, deciduous, curly; outer ligule an irregular glabrous rim up to 0.02 mm long; inner ligule 0.02–0.03 mm long, truncate, minutely ciliate; pseudopetioles ca. 0.5 mm long; blades 4.8–6.2 cm long, 2–3 mm wide, linear to linear-lanceolate, adaxially and abaxially green

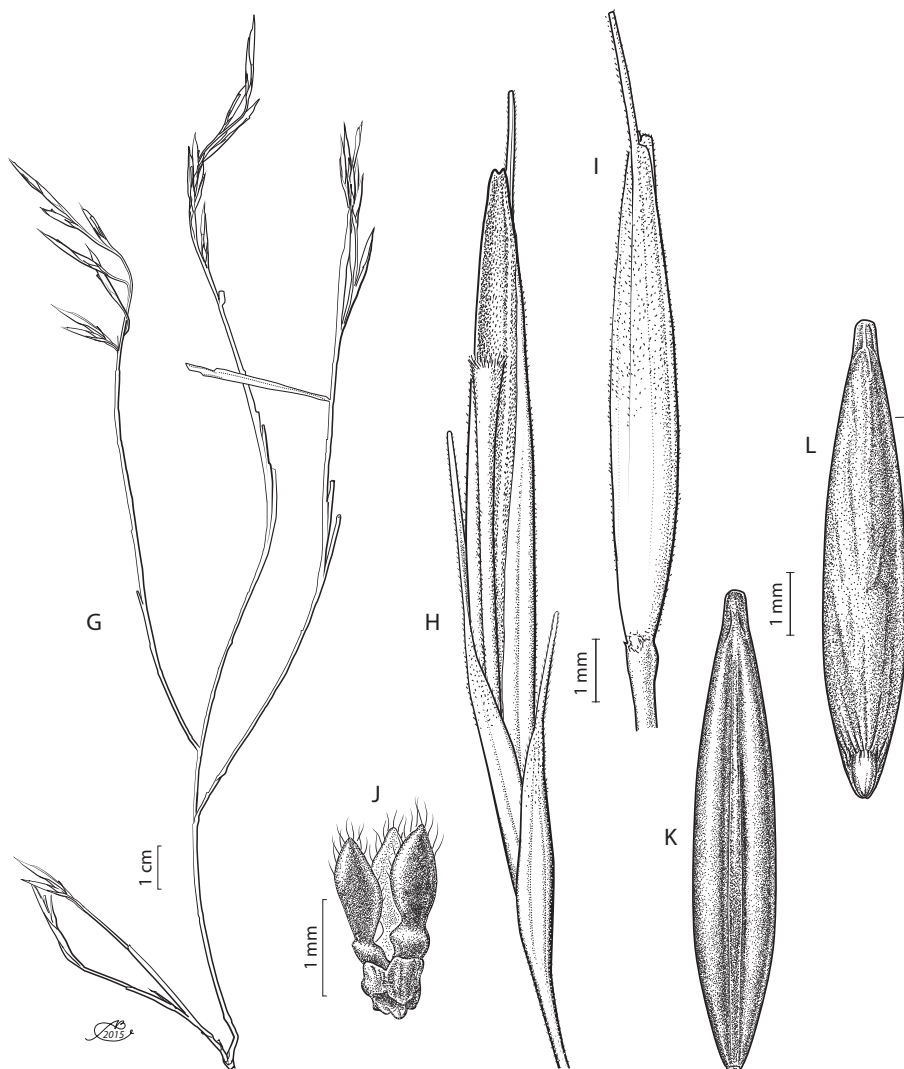


Fig. 5. *Otatea rzedowskiorum*. G. Flowering branch. H. Spikelet ventral view, glabrous awned glume I, rachilla joint with pubescent apex, palea with glabrous keels, scabrous-pubescent sulcus and bifid apex and ventral view of awned lemma. I. Floret dorsal view, showing scabrous awned lemma. J. Anterior glabrous lodicules pair, abaxial view and adaxial apex of the posterior one. K. Caryopsis hilum. L. Caryopsis embryo. Based on E. Ruiz-Sanchez, M.A. Perez Farrera & W. Wysocki 467.

and glabrous, abaxially, without a patch of white cilia, the base attenuate, the apex acute, the margins weakly serrulate for one side. Synflorescences not seen.

3.4.7. Habitat and distribution

This species is only known from two populations in the state of Hidalgo, Mexico in the locality known as Barranca de Tolantongo, a protected natural area. *O. victoriae* inhabits xerophilous scrub on calcareous soils from 1700 to 2140 m a.s.l. in elevation, with a mean annual temperature of 17.4 °C, and an annual precipitation of 352 mm.

3.4.8. Etymology

The specific epithet honors Dr. Victoria Sosa, my former Ph.D. advisor, in recognition of her productive career in botany.

3.4.9. Phenology

This species has never been collected with flowers.

3.4.10. Additional specimens examined

MEXICO. Hidalgo, Barranca de Tolantongo, 2140 m a.s.l., 3 January 1977, *Medrano & Hiriart 10272* (MEXU); Tolantongo Ravine, La Corona Hill, 1 July 1977, *Medrano & Pontet 10553*

(MEXU); Tolantongo Ravine 13.6 km to the ravine from Tolantongo de El Cubo, 1735 m a.s.l., 18 October 2005, *E. Ruiz-Sanchez & P. Carrillo-Reyes 114* (XAL); at 6 km from the junction with Dos Rios-Metzontla down from the Tolantongo grottos 1764 m a.s.l., 12 July 2014, *E. Ruiz-Sanchez, V. Sosa & E. Gándara 479* (IEB, XAL).

3.4.11. Species comparison

On the basis of vegetative morphology *O. rzedowskiorum* is similar to *O. ramirezii*, and *O. victoriae*. The species are similar in habit with erect culms and the foliage leaves are similar in size and form. However, *O. ramirezii* develops one single branch per node, whereas *O. victoriae* develops three unequal branches per node, the central one is twice as wide as the laterals and *O. rzedowskiorum* develops three subequal branches per node. Fimbriae in culm leaves grow from two irregular auricle-like structures at the summit of the sheath on both sides in *O. rzedowskiorum*, while the auricle-like structures are lacking in *O. ramirezii* and *O. victoriae*. Foliage leaves in *O. rzedowskiorum* are similar in size to those of *O. ramirezii* (8–11 vs. 9–11) with patch of the white cilia on the abaxial side and larger than *O. victoriae* (4.8–6.2), lacking patch of white cilia on the abaxial side. However *O. rzedowskiorum* has wider foliage leaves than *O. ramirezii* and *O. victoriae* (4–12 mm

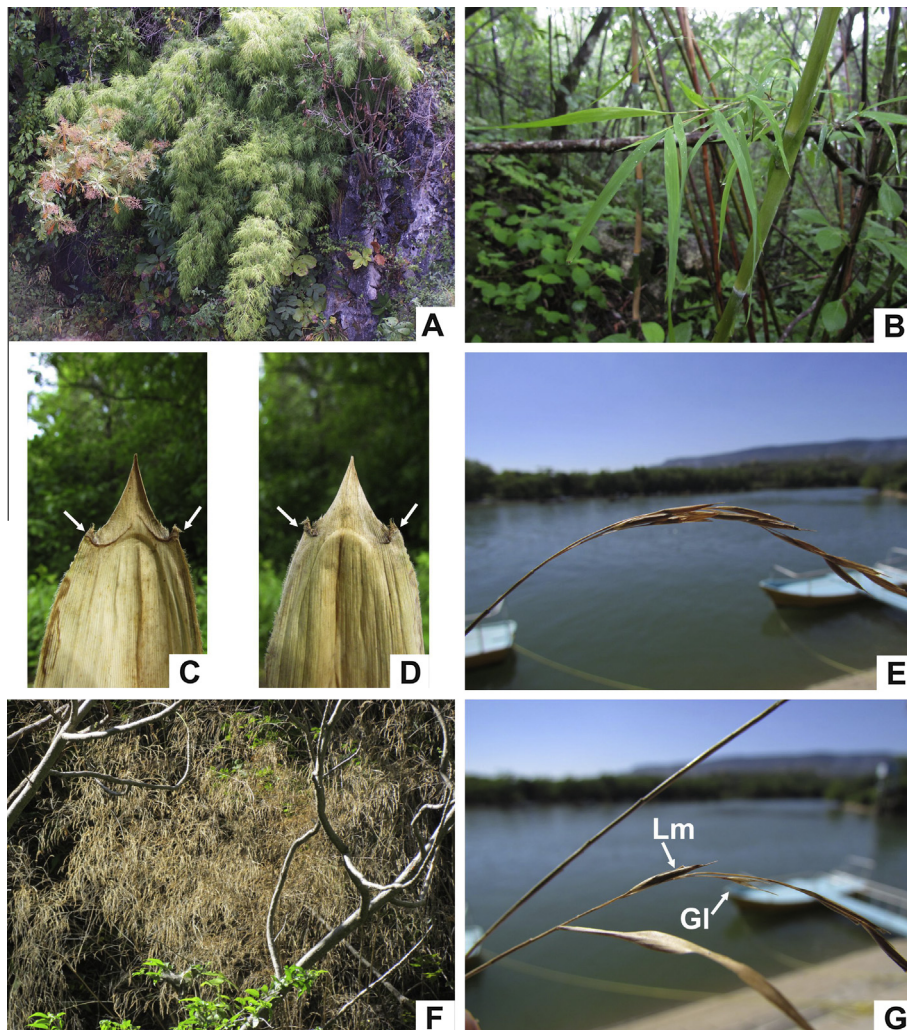


Fig. 6. *Otatea rzedowskiorum* A. Sterile clump of *O. rzedowskiorum* in the glen bordering tropical dry forest (2005). B. New shoot and foliage leaf complements. C. Culm leaf adaxial view, with arrows indicating the auricle-like structure with fimbriae. D. Culm leaf abaxial view, with arrows indicating the auricle-like structure with fimbriae. Note the continuous growth between the sheath and the blade. E. Synflorescence showing a few spikelets. F. Fertile culm flowering (2014). G. Spikelet, Lm = awned lemma, Gl = awned glumes.



Fig. 7. *Oatea victoriae*. A. Culm fragment showing culm leaves. B. Culm leaf abaxial view. C. Culm leaf adaxial view. D. Branch complement showing three unequal branches. E. Branch, showing secondary branches and foliage leaves. F. Ligular area of foliage leaf, showing fimbriae. Based on E. Ruiz-Sanchez, E. Gándara & I. Gúzman 484.

vs. 3–4 mm; 2–3 mm) (Table 1). Foliage sheaths are glabrous in *O. ramirezii* and *O. rzedowskiorum* and scabrous in *O. victoriae*. Fimbriae on foliage leaves are curly and scabrous in *O. victoriae*, curly and glabrous in *O. ramirezii* and erect and glabrous in *O. rzedowskiorum* (Table 1). Synflorescences in *O. rzedowskiorum* develop 4–6 spikelets and *O. ramirezii* develop 6–25 spikelets. Spikelets in *O. rzedowskiorum* are 1.5–2.5 cm long, but longer in *O. ramirezii*: 2.5–3 cm long. Glumes are glabrous in *O. rzedowskiorum*, but scabrous in *O. ramirezii* (Table 1). The differences between *O. rzedowskiorum* and *O. acuminata* are: culm leaves are glabrous in *O. rzedowskiorum* and pubescent with irritating stiff dark hairs in *O. acuminata*, culm leaf blades are fused-persistent in *O. rzedowskiorum* and free-deciduous in *O. acuminata*. Oral setae in culm leaves are absent in *O. rzedowskiorum* and usually present in *O. acuminata*. Fimbriae on culm leaves are borne in two irregular auricle-like structures in *O. rzedowskiorum* and at the shoulders in *O. acuminata*. Foliage leaf blades are smaller and thinner (6.5–11.5 cm; 3–4 mm) in *O. rzedowskiorum* and longer and wider (10–22 cm; 5–12 mm) in *O. acuminata* (Table 1). The differences between *O. victoriae* and *O. acuminata* are: culm leaves are glabrous in *O. victoriae* and pubescent with irritating stiff dark hairs in *O. acuminata*, culm leaf blades are fused-persistent in *O. victoriae* and free-deciduous in *O. acuminata*. Oral setae in culm leaves are absent in *O. victoriae* and usually present in *O. acuminata*.

Branches per node are 3 unequal, the central borne first and double the size of the two laterals in *O. victoriae* and 3 subequal in *O. acuminata*. Foliage leaf blades are smaller and thinner (4.8–6.2 cm; 2–3 mm) in *O. victoriae* and longer and wider (10–22 cm; 5–12 mm) in *O. acuminata*. Patch of white cilia on abaxial side is absent in *O. victoriae* and present in *O. acuminata* (Table 1).

4. Discussion

Ruiz-Sanchez and Sosa (2010) in their species delimitation study using cpDNA + nrDNA found in their phylogeny that populations 16 and 25, corresponding to *Oatea* Hidalgo and *Oatea* Chiapas, are nested in two different, well supported clades (see Fig. 2 in Ruiz-Sanchez and Sosa, 2010). A different study by Ruiz-Sanchez et al. (2011a) using only cpDNA markers, reported that the same populations of *Oatea* from Hidalgo and Chiapas (coded as *O. acuminata* 114 and *O. acuminata* 119) were again nested in two different, well supported clades (see Fig. 1 in Ruiz-Sanchez et al., 2011a). Those two different analyses supported *O. acuminata* as a polyphyletic species. In this study using BI inference from one nuclear and five chloroplast markers, plus morphological characters, I obtained the same result (Fig. 2a): *O. acuminata* is a polyphyletic species and *O. acuminata* Hidalgo and *O. acuminata*

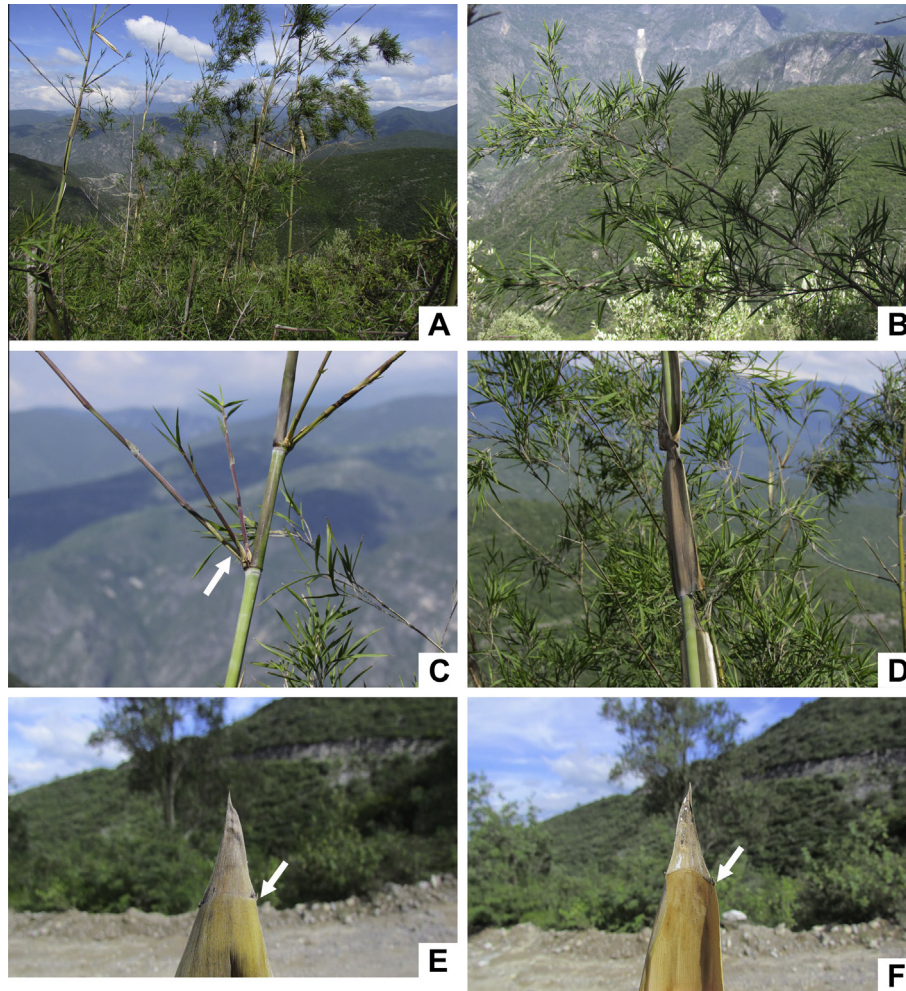


Fig. 8. *Oatea victoriae*. A. Clump of *O. victoriae*, showing new culms. B. Culm, showing branches with new leaves. C. Development of new branches. The white arrow shows the three main branches; the central one develops before the laterals and is twice as wide as the laterals are. D. Culm leaves at mid-culm showing intravaginal branch pattern. E. Culm leaf abaxial view, white arrow indicates fimbriae. Note the continuous growth between the sheath and the blade. F. Culm leaf adaxial view, white arrow indicates fimbriae.

Chiapas are well supported as different evolutionary lineages based on the results of the non-parametric method of Wiens and Penkrot (2002) for delimiting species (Figs. 1 and 2a).

The BP&P analyses used a resolved guide tree to estimate speciation events (Yang and Rannala, 2010), however I obtained an unresolved position for *O. carrilloi* (Fig. 2a). To solve this problem Leaché and Fujita (2010) recommended the use of a guide tree with resolved polytomies. I fitted *O. carrilloi* sister to *O. glauca* following the morphological tree analysis of Ruiz-Sanchez and Sosa (2010). Leaché and Fujita (2010) also suggested incorporating separate prior probability distributions for the ancestral population sizes (θ) and for the root age (τ_0), because these benefit from the Bayesian species delimitation method. In this study I used three different combinations of priors, leading to the combination of θ $G(2, 2000)$ and τ_0 $G(2, 2000)$ having the highest probabilities of speciation events. This combination means that the speciation events of *Oatea* were driven by small ancestral population sizes and shallow divergence. Flowering cycles in *Oatea* species are not annual, but rather are long and last up to 30 years (Ruiz-Sanchez et al., 2011b; Ruiz-Sanchez, 2013), resulting in long generation times and this is reflected by the shallow divergence times. Additionally, the complex geological history of Mexico resulting from volcanic activity occurring mainly in central Mexico (Gómez-Tuena et al., 2007; Ferrari et al., 2012) isolated plant

populations (Ruiz-Sanchez and Specht, 2014). Owing to the small populations of *Oatea* species this, in combination with its long flowering cycles, allowed speciation to occur. Speciation in *Oatea* took place from the Late Miocene to the Late Pleistocene and coincides with the volcanic activity of the Trans-Mexican Volcanic Belt (Gómez-Tuena et al., 2007; Ferrari et al., 2012) for the species distributed in central Mexico.

In a previous molecular dating analysis by Ruiz-Sanchez (2011) the divergence time estimate indicated that *Oatea's* origin was later (4.09–0.33 Ma) than the time obtained in the present analysis (10.15–2.73 Ma, Fig. 3). A new molecular dating study using eight chloroplast and two nuclear markers (Ruiz-Sanchez and Sosa, 2015), suggests slightly older divergence times for *Oatea* (10.5–4.4 Ma). Ho and Duchêne (2014) mention that an accurate inference depends on the sequence data, model selection and reliable calibrations. The sequence matrix of Ruiz-Sanchez (2011) consisted of a single chloroplast marker and one secondary calibration point, while this new inference is based on one chloroplast and one nuclear marker and five calibration points, making this divergence time estimate more accurate.

The non-parametric method of Wiens and Penkrot (2002) applied to the *Oatea* matrix (one nrDNA, five cpDNA markers and morphological data) recognized nine evolutionary lineages from which *O. acuminata* from Chiapas and *O. acuminata* from

Table 1Comparison of morphological characters among *Otatea* species: *O. acuminata*, *O. ramirezii*, *O. rzedowskiorum* and *O. victoriae*.

Character/taxon	<i>O. acuminata</i>	<i>O. ramirezii</i>	<i>O. rzedowskiorum</i>	<i>O. victoriae</i>
<i>Culm habit</i>	Erect and arching apically	Erect	Erect to slightly apically arching	Erect
<i>Culm size</i>				
Length (m)	2–10	2–5	1.5–4.5	2–5
Diameter (cm)	1–5	0.8–2.5	0.8–1.5	1–2
<i>Internode</i>				
Length (cm)	15–24	12–16	10–17.5	6.5–31
Consistency	Hollow or solid	Hollow	Solid	Hollow
Wall thickness (mm)	2–4	3–5	–	2–4
<i>Culm leaves</i>				
Sheath length (cm)	10–30	10–17	8–12	6.5–13.8
Relative position	Overlapping or not	Non-overlapping	Non-overlapping	Non-overlapping
Duration	Deciduous or persistent	Deciduous	Deciduous	Deciduous
Abaxial surface indument	Irritating stiff dark hairs present	Glabrous	Glabrous	Glabrous
<i>Culm leaf blades</i>				
Length (cm)	6–18	1.8–4.5	2.5–7.5	0.8–7
Position	Erect	Erect	Erect	Erect
Margin indument	Ciliate, glabrous or hirsute	Glabrous	Ciliate	Minutely ciliate
Duration	Free, deciduous	Fused, persistent	Fused, persistent	Fused, persistent
<i>Oral setae on culm leaves</i>				
Length (mm)	10–15 when present	Absent	Absent	Absent
Color in living specimens	Brown	–	–	–
<i>Fimbriae on culm leaves</i>	Present	Present	Present	Present
Position	At shoulders	At shoulders	Borne in two irregular like-auricles	At shoulders
Length (mm)	4–7	1–3	3–5	1.5–2
<i>Branches per node</i>	3 subequal	1 (rarely 2)	3 subequal	3 unequal (central branch appears first and is twice the size of the two lateral branches)
<i>Foliage leaf blade</i>				
Length (cm)	10–22	6.5–12.5	6.5–11.5	4.8–6.2
Width (mm)	5–12	4–12	3–4	2–3
Patch of white cilia in the abaxial side	Present	Present	Present	Absent
<i>Fimbriae on foliage leaves</i>				
Length (mm)	1–3	1–2	1–2	0.5–1
Posture	Curly	Curly	Erect	Curly
<i>Habitat</i>	Tropical dry forest and xerophilous scrub	Tropical dry forest and ecotone with oak forest	Tropical dry forest	Xerophilous scrub
<i>Distribution</i>	Widespread throughout Mexico	Queretaro, and Hidalgo (Mexico)	Chiapas (Mexico)	Hidalgo (Mexico)

Hidalgo can be recognized as distinct species separate from *O. acuminata* s.s. However the BP&P only supports five speciation events for the three previously described species (*O. acuminata*, *O. carrilloi* and *O. glauca*) and one non-described species *O. acuminata* Hidalgo, and the speciation event of *O. fimbriata*–*O. ximena* and *O. acuminata* Chiapas. Incongruent results from the two methods (parametric and non-parametric) used for species delimitation have been reported in different studies. Carstens et al. (2013) in their Table 2 summarized studies in which researchers found incongruencies in species delimitation using different methods. Carstens et al. (2013) pinpointed violations of the methods' assumptions as the possible source of the incongruent results. A small sample size and limited number of loci used could lead to incorrect results in coalescent-based methods (Fujita et al., 2012; Carstens et al., 2013). Here I used six loci, however the five cpDNA markers used are considered to be a unit because they are linked and one of the assumptions for the coalescent-based species delimitation methods is to use unlinked multiloci (Fujita et al., 2012). It is possible that one nuclear and five chloroplast markers are not enough to detect coalescent speciation events, at least in *Otatea*. However, the use of morphology in combination with molecular data for the non-parametric analysis increased the nodal support for the phylogenetic tree (Pyron, 2015). In the

other hand, the morphological approaches should be necessary for the description of new species (Fujita et al., 2012). The implementation of nuclear orthologous single copy genes in phylogenetic study of bamboos has already begun (Triplett et al., 2014; Zhang et al., 2014). In the near future using of single copy nuclear genes applied to species delimitation in woody bamboos along with coalescent-based methods will make it possible to finally identify coalescent speciation events or potential past hybridization events in these fascinating plants.

Taking into account the results obtained using both the parametric and non-parametric methods I conclude that *O. acuminata* Chiapas and *O. acuminata* Hidalgo should be recognized as separate evolutionary lineages, formally described here as *O. rzedowskiorum* from Chiapas and *O. victoriae* from Hidalgo. Phylogenetically, *O. victoriae* is more closely related to *O. acuminata* s.s., and *O. rzedowskiorum* is more closely related to *O. fimbriata* and *O. ximena* (Fig. 2a and b). Morphologically, both new species are different from *O. acuminata* and from the other species of *Otatea* (Table 1). Characters of the culm leaves are used for identification to both the genus and species levels. Culm leaves are glabrous in *O. rzedowskiorum* and *O. victoriae*, but are pubescent with irritating stiff dark hairs in *O. acuminata*. Culm leaf blades are fused and persistent in *O. rzedowskiorum* and *O. victoriae*, and free and

deciduous in *O. acuminata*. There are three main subequal branches per node in the three species, however in *O. victoriae* the central one appears first and is two times wider than the laterals. The characters of *O. acuminata*, *O. ramirezii*, *O. rzedowskiorum* and *O. victoriae* are compared in Table 1.

O. acuminata grows in tropical dry forest or in the ecotone between tropical dry and oak forests on the Pacific slope as well along the Trans-Mexican Volcanic Belt to Veracruz (Fig. 1). Speciation along the Trans-Mexican Volcanic Belt is mainly due to the uplift of this mountain range and is related to habitat fragmentation and the resulting isolation of animal and plant populations (Bryson et al., 2011a,b; Bryson and Riddle, 2012; Ruiz-Sanchez and Specht, 2014). Uplift could be responsible for the isolation and speciation of both *O. victoriae* and also that of *O. transvolcanica* (Fig. 1). *O. victoriae* populations only grow in glens of xerophilous scrub on calcareous soils from 1700 to 2140 m a.s.l., in elevation where mean annual temperature is 17.4 °C and annual precipitation is 352 mm (<http://smn.cna.gob.mx/climatologia/Mensuales/hgo/00013105.TXT>). On the other hand *O. rzedowskiorum* grows in tropical dry forest on calcareous soils from 600 to 1000 m a.s.l., in elevation where mean annual temperature is 26.7 °C and annual precipitation is 973.6 mm (<http://smn.cna.gob.mx/climatologia/Mensuales/chis/00007134.TXT>). This species is separated from northern *Otatea* species by the Isthmus of Tehuantepec (Fig. 1). The recent study by Ornelas et al. (2013) showed that the Isthmus of Tehuantepec could be a barrier to gene flow among the populations of animals and plants distributed on either side of the isthmus, allowing speciation to occur by vicariance.

5. Concluding remarks

The use of molecular and morphological data and applying parametric and non-parametric methods has proven be useful for species delimitation in *Otatea*. Both methods recognized two new species formerly known as *O. acuminata*, however the parametric method was sensitive to the small sample size and the limited loci used here. On the other hand, when analyzed using the non-parametric method, the molecular and morphological characters I selected resulted in increased nodal support and the morphological recognition of two new *Otatea* species here described as *O. rzedowskiorum* and *O. victoriae*. The uplift of the Trans-Mexican Volcanic Belt and the formation of the Isthmus of Tehuantepec played an important role in the speciation of *Otatea* by isolating populations and limiting gene flow.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.08.004>.

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