FACULTY OF SCIENCES

Phylogenetic incongruence in Oxandra

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Contents

Introduction

Phylogenies form the framework for any kind of evolutionary research, as without a phylogeny it would be impossible to talk about important concepts such as ancestral characters, synapomorphies,… which in turn are essential to gain insight in e.g. genome evolution or temporal aspects of evolution, such as when certain traits were acquired. Also in ecology the incorporation of phylogenetic information is becoming more important (e.g. Weber and Agrawal, 2012). Due to this importance, researchers have looked for ways to infer and improve phylogenetic trees, in order to better understand the relationships between different organisms. The most direct way is looking at the morphology of taxa, following the basic logic that the more taxa resemble each other, the more closely related they are. This has for example been done in the Annonaceae, a tropical family belonging to the Magnoliids (APG IV, 2016). Doyle and Le Thomas (1994) inferred phylogenetic relationships within this family based on morphology, using characters ranging from habit and phyllotaxy to the infratectal structure of the pollen. In a follow-up study they focused more on the relationships between the major clades in Annonaceae (Doyle and Le Thomas 1996), which still holds up decently well with recent phylogenies (compare e.g. with Chatrou et al. 2012). Even today phylogenies based on morphology are still made (Lopes and Mello-Silva 2019), for example to compare with molecular data or to aid in recognition and determination of species. The benefit of these phylogenies is that they can offer practical characters that can be used to determine synapomorphies: characters that are unique to clades and thus can be used to define them.

However, in the last three decades phylogenetics has been completely turned over as it became easier and cheaper to sequence DNA. DNA sequencing allows the use of far more characters then would ever be possible using morphological characters. In Annonaceae, the first study at the genus level based on molecular data was done by Bygrave (2000) using only a single gene, *rbcL*. Since then more phylogenetic trees of (parts of) Annonaceae have been made, using more and more markers as sequencing became better and cheaper (Mols et al. 2004; Richardson et al. 2004; Pirie et al. 2006; Couvreur et al. 2011; Chatrou et al. 2012; Guo et al. 2017; Lopes et al. 2018). Interestingly, all these studies use only markers from the chloroplast genome (sometimes also called the plastome). Only few studies considering Annonaceae phylogenetics make use of markers of the nuclear genome such as the ribosomal DNA (Couvreur et al., 2019; Hoekstra et al., 2017) or microsatellite flanking regions (Chatrou et al., 2009). This discrepancy is caused by the fact that the use of chloroplast markers has several advantages over using nuclear or mitochondrial markers. The chloroplast genome is abundant and relatively small which allows for easy extraction. Furthermore, most genes are single-copy while nuclear genes are often member of gene families, meaning there are multiple paralogues or even pseudogenes present with different and sometimes very divergent sequences. Lastly, different chloroplast genes evolve at different speeds, making them useful to obtain phylogenies at the species, genus, family level or even higher (Soltis & Soltis, 1998).

Despite the obvious benefits of the chloroplast genome for phylogenetics, it can be beneficial to also include nuclear or mitochondrial markers, not only to obtain more data but also to be able to compare the phylogenetic trees based on the different plant genomes. The nuclear

genome also contains markers that are rapidly evolving and can be used to understand relationships among closely-related species or populations. It has been shown that phylogenetic trees based on chloroplast data might differ from trees based on mitochondrial or nuclear data, a phenomenon known as phylogenetic incongruence. Examples have been found at the species level (de Sousa et al., 2016) genus level (Cristina Acosta & Premoli, 2010; Galbany-Casals et al., 2014; Spooner et al., 2017), at the tribal level (Pelser et al., 2010) and even at higher levels (Sun et al., 2015). If not caused by technical difficulties or mistakes such as contamination, this incongruence can have different origins such as hybridization (Spooner et al., 2017; Sun et al., 2015), chloroplast capture (Cristina Acosta & Premoli, 2010), incomplete lineage sorting or horizontal gene transfer (Wendel & Doyle, 1998).

In the Annonaceae most genera are monophyletic, but in some cases relationships within and between genera are still unsolved (Guo et al., 2017). One notorious case is the genus *Oxandra*, consisting of 27 species (Junikka et al., 2016). It occurs in the Neotropics from the states Nayarit and Veracruz in Mexico to Rio de Janeiro in Brazil. Most species are trees, although some shrubs occur as well (Junikka et al., 2016). It is part of the tribe Malmeeae (Chatrou et al., 2012). Although the tribe itself is clearly defined, the relationships between genera are far from clear (see for example Chatrou et al. 2012; Pirie et al. 2006) and especially the monophyly of *Oxandra* is difficult to demonstrate or refute. Already in 2004 it was recovered as non-monophyletic, albeit with low support values, (Richardson et al. 2004) and since then no study has ever recovered *Oxandra* as monophyletic (Chatrou et al., 2012; Guo et al., 2017; Lopes et al., 2018; Pirie et al., 2006). In the two latest studies, two separate clades were found, with a few *Oxandra* species not belong to either of those (Guo et al., 2017; Lopes et al., 2018). Still, it is interesting to note that *Oxandra* was originally described as a natural and sharply delimited genus (Fries (1931) as cited in Junikka et al. 2016), something which is not at all reflected in the molecular data. Could it be that, despite considerable advancements in sequencing technologies, there still are insufficient data to resolve *Oxandra* as monophyletic? Or is morphology misleading us and is *Oxandra* really not monophyletic?

As mentioned above, all studies recovering *Oxandra* as non-monophyletic used only chloroplast data. New, preliminary research comparing chloroplast, nuclear and mitochondrial data shows an interesting pattern: the phylogenetic tree based on chloroplast data shows the familiar pattern with two clades and a few separate species. The phylogenies based on nuclear and mitochondrial data are similar but differ from the chloroplast one: the two clades are recovered as well, but are sister clades, rendering at least a big part of *Oxandra* as monophyletic. Not all *Oxandra* species were sampled, and at least *O. sessiliflora* and *O. unibracteata* were not placed in one of the two clades. It is still unknown what is causing the phylogenetic incongruence between chloroplast data on the one hand and nuclear and mitochondrial data on the other hand. Also unknown is if and how certain morphological traits align with the different phylogenies. Such morphological traits can be traits that are used in identification (e.g. raised or sunken primary vein on the upper side of the leaves (Junikka et al., 2016)) but have little impact on life history. But maybe more biologically relevant are traits that influence reproduction. One example in *Oxandra* is the occurrence of androdioecy: an uncommon type of reproduction system involving both hermaphroditic and male plants co-occuring in the same species. It has evolved independently only a few times in plants (Renner,

2014). Androdioecy has been suggested to be an intermediate step from hermaphroditism (bisexual flowers) to dioecy (reviewed in Pannell 2002), but in fact most empirical examples show it evolved from dioecy (Divyasree & Raju, 2019; Krähenbühl et al., 2002; Pannell, 2002). An extra factor to take into account is that some species are functionally dioecious. This means that the bisexual flowers of an apparent androdioecious species are defect in their male function (e.g. inviable pollen), making the species functionally dioecious (reviewed in Charlesworth 1984). Although androdioecy is generally very rare, it has evolved a few times independently in the Annonaceae (Saunders, 2010). Especially in the tribe Malmeeae it occurs frequently, in genera such as *Klarobelia, Ephedranthus, Pseudomalmea, Pseudephedranthus* and *Pseudoxandra* (Lopes et al. 2018). In *Pseudoxandra spiritus-sancti* 'true' androdioecy was recently confirmed, as the bisexual flowers possess functional stamens, making the species functionally androdioecious (Lopes et al. 2018).

Four species in *Oxandra* have been described as androdioecious: *O. maya*, *O. martiana*, *O. mediocris* and *O. panamensis* (Junikka et al., 2016). For Malmeeae and thus for *Oxandra* as well, hermaphroditism is considered plesiomorphic (Lopes et al. 2018) and no dioecious species occur. Therefore, androdioecioecy in *Oxandra* cannot be derived from dioecy nor can they be a step in the transition from hermaphroditism to dioecy, although as mentioned above those are the two explanations most often given to the occurrence of androdioecy. Because of the lack of a well-supported and complete phylogeny of *Oxandra*, it is impossible to assess how many times androdioecy (and other characters) originated here, if it is specific for one of the two clades in *Oxandra*, and what the evolutionary context is.

Objectives

This study tries to gain insight in the origin and evolution of androdioecy in *Oxandra*. This is best doable if a complete phylogeny (or as complete as possible) is constructed. The first objective of this study is to infer phylogenetic relationships of all *Oxandra* species, combined with other genera of the Malmeeae. If the phylogenetic incongruence described above is recovered, we will try to provide an adequate explanation. With this phylogeny, an ancestral state reconstruction of mating system and other relevant characters will be done. With this reconstruction, we will try to gain insight in the evolution of androdioecy in *Oxandra*.

Material and methods

Taxon sampling

As described above an important goal of this study was to provide a phylogeny of *Oxandra* as complete as possible*,* and also include several closely related genera from the tribe Malmeeae. To achieve this, already available accessions were complemented with newly generated data for those *Oxandra* species of which no high-throughput data was available. For the new species, material was gathered either form the herbarium of the Naturalis Biodiversity Center in Leiden (Netherlands) or from silica samples of the Systematic and Evolutionary Botany lab, Ghent University (UDNA, see Table 1). A total of 50 species were used in the analysis, including all 27 *Oxandra* species and a currently unplaced specimen, *Oxandra sp.* (see Junikka et al. 2016, p. 261). Besides all *Oxandra* species, other represented genera are

Cremastosperma, Ephedranthus, Klarobelia, Malmea, Mosanonna, Pseudoxandra, Pseudomalmea and *Ruizodendron,* all part of the tribe Malmeeae.

DNA extraction

DNA extraction for both herbarium and silica samples was done following a modified version of the CTAB (hexadecyltrimethylammonium bromide) extraction protocol (Doyle & Doyle, 1987). Leave samples were ground using a metal bead at 20 Hz until only a fine powder remained. Then 800 µL CTAB, 5µL β-mercaptoethanol, 2.5 µL Rnase (20mg/ml) and 2.5 Proteinase K were added for a total of 810 µL. This mixture was incubated for 3h for herbarium samples and 2h for silica samples, both at 65°C. The aqueous phase was then transferred to 1 volume or 810 µL chloroform:isoamyl alcohol (24:1) and mixed by inverting and gently vortexing. Next, the samples where centrifuged for 5 minutes (12000 rcf) and the resulting aqueous phase was transferred to a new tube containing 1 volume of chloroform:isoamyl alcohol (24:1). After centrifuging 5 minutes (12000rcf) the top aqueous phase with was transferred to a new tube. Corresponding with an approximate volume of 500 µL, 292 µL isopropanol and 40 µL 7.5M AmAc was added. This was incubated for 1h for silica samples and overnight for herbarium samples, both at -20°C. The solution was centrifuged for 30 minutes at 4°C (12000rcf), whereafter the aqueous phase was removed. The remaining pellet was washed with 500 µL 70% ethanol and again centrifuged at 4°C for 10 min. The fluid was removed and the remaining pellet was suspended in 50µL distilled water and stored at -20°C until further use.

	Species	Herbarium collection	Accession number	Material	Extra clean-up	FS protocol	PCR cycles
OX0001	*Oxandra xylopioides* Diels	Chatrou, L. W. 165	UDNA_280	Silica	No	No	4
OX0002	*Oxandra xylopioides* Diels	Pirie, M.D. 30	UDNA_1113	Silica	No	No	4
OX0003	*Oxandra mediocris* Diels	Pirie, M.D. 70	UDNA_1138	Silica	No	No	4
OX0004	*Oxandra mediocris* Diels	Pirie, M.D. 98	UDNA_1159	Silica	No	No	4
OX0025	*Oxandra aberrans* Maas & Junikka	Kennedy, H.; Breedlove, D.E. 1422	U.1605547	Herbarium	Yes	No	7
OX0026	*Oxandra lancealata* (Sw.) Baill.	Fuertes, M. 224	L.4333526	Herbarium	No	No	4
OX0027	*Oxandra leucodermis* (Spruce ex Benth.) Warm.	Liesner, R.L. 6983	U.1610316	Herbarium	No	No	4
OX0028	*Oxandra maya* Miranda	Davidse, G.; Holland, D.L. 36572	U.1090707	Herbarium	No	No	7
OX0029	*Oxandra mediocris* Diels	Alexiades, M.; Pesha, V. 367	U.1605563	Herbarium	No	No	7
OX0030	*Oxandra reticulata* Maas	Coradin, L. et al. 6039	U.1610365	Herbarium	Yes	No	7
OX0031	*Oxandra rheophytica* Maas & Junikka	Callejas Posada, R. et al. 9343	U.1101078	Herbarium	No	Yes	5
OX0032	*Oxandra saxicola* Maas & Junikka	Wood, J.R.I.; Villarroel, D. 25544	L.3728510	Herbarium	No	Yes	5
OX0033	*Oxandra venezuelana* R.E.Fr.	Schatz, G.E.; Janzen, M.I.D. 1088	U.1605572	Herbarium	No	No	4
OX0034	*Oxandra xylopioides* Diels	Neill, D.A.; Palacios, W. 6839	U.1610168	Herbarium	Yes	No	7
OX0035	*Oxandra sp.*	Fernandez, Y. 306	U.1088846	Herbarium	No	No	7

Table 1: Sample preparation details.

All samples were analysed after extraction using a NanoDrop™ 2000 to determine contamination with proteins or leftovers from the extraction. An extra clean-up step was needed for three samples (see Table 1). This was done using SPRIselect beads. An equal volume of beads was added to the sample, mixed and left for 5 minutes. Next, the sample was placed on a magnetic stand for 5 minutes after which the supernatant was removed. The pellet was washed with 200µL 80% ethanol and after 30 seconds the supernatant was removed again. The pellet was dried until it was not shiny anymore (about 30 seconds) and then 40µL water was added. This was mixed and after 2 minutes was placed on the magnetic stand for 5 minutes. Lastly 38 µL was taken from the samples and stored at -20°C until further use.

Library preparation

The size distribution for each sample was checked using gel electrophoresis and ethidium bromide colouring. For two samples a wide range of sizes was visible. To overcome losing too much DNA in the clean-up, for these two samples the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina® was used. This protocol includes an extra step using a fragmentase to standardize the size distribution of the DNA fragments. For all other samples the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® was used. The producer's protocol was followed; the size selection using purification beads was done using 30 µL and then 15 µL, which yields an approximate insert size of 250 bp. To determine the amount of PCR cycles the concentration of the samples was measured using a Qubit™ dsDNA BR Assay Kit, then following the producer's protocol for the number of cycles (see Table 1). After the PCR and the last clean-up step, the sample concentration was measured again to be able to perform equimolar pooling. In the final pool all samples had a concentration of 1.159 nM. The sequencing was performed by BGI Genomics on DNBSeq™, generating paired-end 150bp reads. This will allow us to obtain sequences of the high-copy portion of the genome, a technique called genome skimming (Straub et al., 2012) which has been show to work well with herbarium specimens (Alsos et al., 2020; Bakker et al., 2016). This high-copy fraction consists of the entire plastome, the nuclear ribosomal DNA and the mitochondrial DNA.

Genome assembly and phylogenetics preparation

To ensure all data were subjected to the same procedure, raw Illumina data was used for each accession. The assembly was done using GetOrganelle v1.7.4-pre2 (Jin et al., 2018) as it was considered best in a comparison between chloroplast assembly tools (Freudenthal et al., 2019) and it can also be used to assemble nuclear data.

Unfortunately, due to covid-19 and time constraints, it was not possible to continue working with the newly generated data. Already available data was used instead, which was obtained using the extraction and library preparation method as described above. Genome assembly was done using IOGA (Bakker et al., 2016). Two already prepared matrices were used for further analysis, one for the chloroplast counting 33 species and 61136 bp in total, and one for the nuclear ribosomal DNA consisting of 32 species with a total length of 5826 bp. Both matrices were analysed separately using maximum likelihood and Bayesian methods. First, PartitionFinder v2.1.1 (Lanfear et al., 2017) was used to determine the best partition scheme for both matrices. For the chloroplast matrix branch lengths was set to linked, the model was GTR+G, the criterium for model selection was BIC and the search algorithm was 'greedy'. Each spacer, intron and protein coding genes separated by codon was inputted as a data block. To overcome crashes the *petB* exon 1 and 2, and the *rps16* exon 1 and 2 were combined together. For the nuclear matrix, the branch lengths were set to linked, the model was 'MrBayes' (this makes it so that only models available in MrBayes can be selected), the criterium for model selection was BIC, the search algorithm was set to 'greedy' and the input data blocks were the three rRNA sequences and both ITS sequences. Using these settings, 8 data blocks were found for the chloroplast matrix and the nuclear matrix was partitioned into 3 data blocks.

Maximum likelihood analysis

The maximum likelihood analysis was performed using RAxML v8.2.12 (Stamatakis, 2014) at the CIPRES Science Gateway (Miller et al., 2011). A rapid bootstrapping with 100 iterations was performed after which the maximum likelihood search was performed in a single run.

Bayesian analysis

The Bayesian analysis was performed using MrBayes v3.2.7a (Ronquist & Huelsenbeck, 2003) at the CIPRES Science Gateway. Four runs with four chains running for 20 million generations were done for both datasets. Temperature was set to 0.1 and after the analysis a burnin fraction of 0.25 was determined. The convergence was checked using Tracer v1.7.1 (Rambaut et al., 2018). ESS values for all parameters were above 7000 for both datasets, showing that the analyses ran sufficiently long. For each dataset, a 50% majority rule consensus tree was obtained with posterior probabilities for each node.

Ancestral state analysis

Ancestral state reconstruction was carried out for three characters: mating system, primary vein placement and seed rumination. Mating system was chosen because, as said above, it is the focus of this study. The two other characters were chosen because of their importance in species identification (see e.g. Junikka et al. 2016). These three characters were scored for all genera in the phylogenetic trees obtained above, using the following sources: *Oxandra* (Junikka et al., 2016), *Cremastosperma* (Pirie et al., 2018), *Ephedranthus* (Lopes and Mello-Silva 2019), *Klarobelia*, *Mosannona*, *Pseudomalmea* (all Chatrou 1998), *Malmea* (Chatrou 1997), *Pseudoxandra* (Maas & Westra, 2003) and *Ruizodendron* (Erkens et al., 2017). For some species, there was intraspecific variation in the rumination of the seed. These were coded as polymorphic characters (e.g. spiniform and peg-shaped (1 + 2) in the same species).

	0	1	2
Mating system	Hermaphroditic	Androdioecious	
Primary vein	impressed/flat	raised	
Ruminations	Lamellate	spiniform	peg-shaped

Table 2: Characters states used in ancestral state reconstruction.

Ancestral state analyses were all performed in Mesquite v3.61 (Maddison & Maddison, 2019), for both datasets (chloroplast and nuclear) using the phylogeny based on Bayesian interference as described above. For the mating system and the primary vein placement, a maximum likelihood reconstruction was done. As it is not possible to perform maximum likelihood reconstruction in Mesquite if polymorphic characters are present, reconstruction of the ruminations was done using maximum parsimony.

Results

Phylogenetic relationships

A total of four phylogenetic analyses were conducted: both the chloroplast and the nuclear ribosomal DNA were analysed separately using maximum likelihood Bayesian statistics. The chloroplast phylogeny shows an almost completely resolved tree (Figure 1), while the nuclear phylogeny shows some polytomies (Figure 2). In general, posterior probabilities were higher than bootstrap values. In both the nuclear and the chloroplast phylogeny, two clades containing most *Oxandra* species were recovered: Clade 1 consists of *O. guianensis, O. laurifolia, O. panamensis, O. macrophylla, O. sphaerocarpa* and *O. martiana*. Clade 2 consists of *O. surinamensis, O. longipetala, O. krukoffii, O. riedeliana, O. euneura, O. polyantha, O. asbeckii, O. espintana* and *O. bolivarensis*. In both phylogenies, both clades receive maximum support from posterior probabilities and bootstrap values. Two *Oxandra* species did not belong to either clade: *O. sessiliflora* and *O. unibracteata*. However, the position of the two clades differs between both phylogenies. The nuclear phylogeny shows both clades as sister clades, with maximum support, which would make *Oxandra* mostly monophyletic (except for *O. sessiliflora* and *O. unibracteata*). On the contrary, the chloroplast phylogeny clearly shows *Oxandra* as polyphyletic, with both clades separated from each other. Three species were sampled twice: *Oxandra guianensis, O. asbeckii* and *O. espintana*. *O. guianensis* and *O. asbeckii* are recovered as monopyletic with maximum support, while *O. espintana* seems to be polyphyletic as *O. bolivarensis* is nested with the clade containing both *O. espintana* accessions.

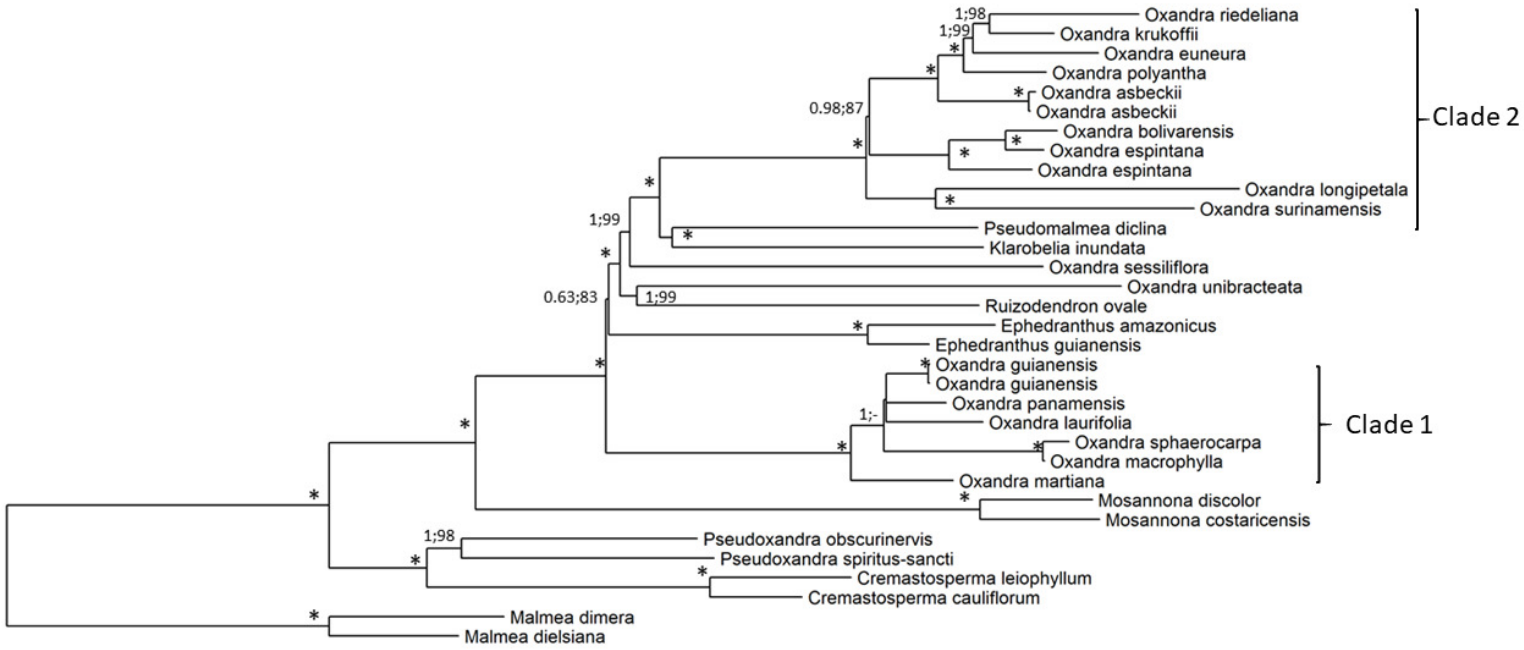

Figure 1: Plastid phylogeny based on Bayesian 50% majority consensus rule. Node labels show posterior probabilities and bootstrap values. Asterisk means full support for Bayesian and maximum likelihood statistics, - means the clade was not recovered in the analysis.

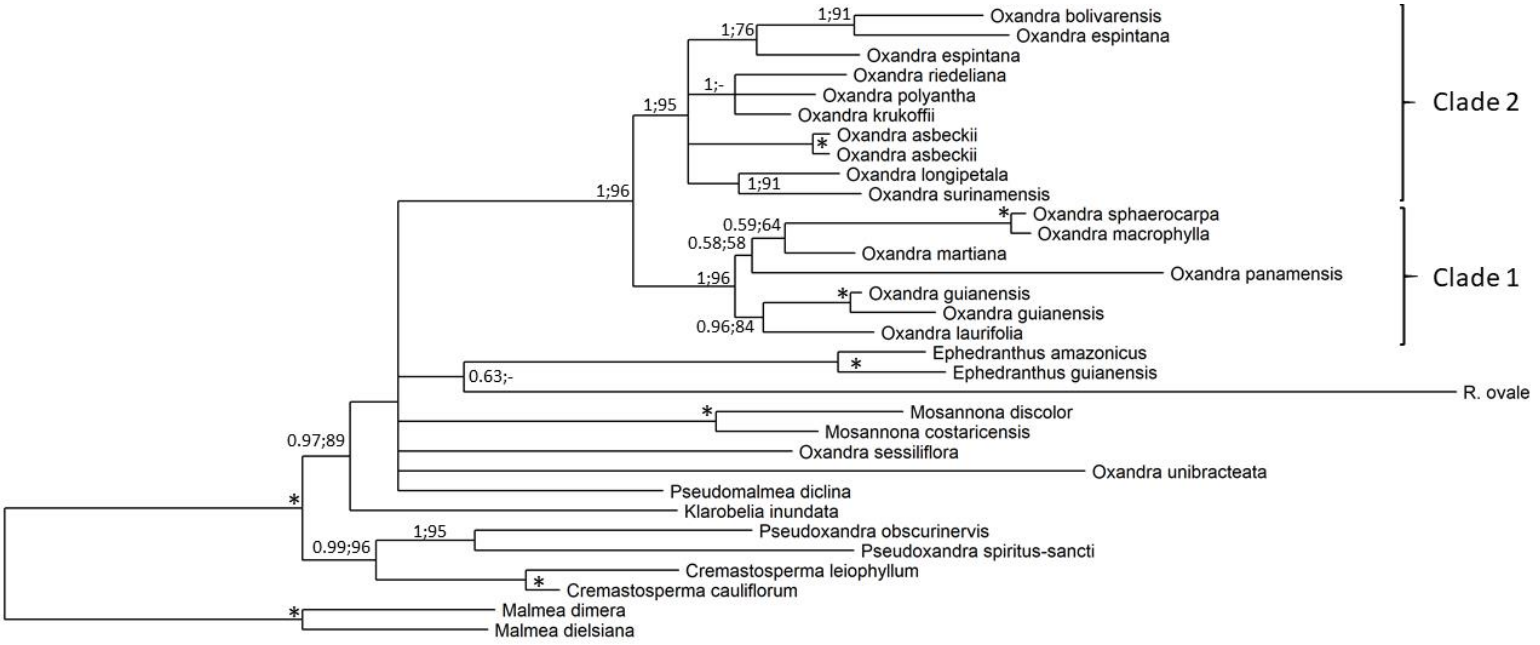

Figure 2: Nuclear phylogeny based on Bayesian 50% majority consensus rule. Node labels show posterior probabilities and bootstrap values. Asterisk means full support for Bayesian and maximum likelihood statistics, - means the clade was not recovered in the analysis.

Ancestral state reconstruction

Ancestral state reconstruction was performed for the plastid and the nuclear phylogeny for three characters: mating system, primary vein placement and seed rumination. Two states were considered for mating system: hermaphroditism, which is plesiomorphic for the tribe Malmeeae (Lopes et al. 2018) and androdioecy. Androdioecy occurs four times in *Oxandra* (Junikka et al., 2016), but only *O. martiana* and *O. panamensis* are sampled in this study. They are both placed in Clade 1. Two closely related species, *Pseudomalmea diclina* and *Klarobelia inundata,* are also androdioecious. According to the plastid phylogeny *P. diclina* and *K. inundata* are successive sister species, but the ancestral state reconstruction shows there is a higher probability that their ancestor was hermaphroditic and that androdioecy originated independently for both species (Figure 3). The nuclear phylogeny doesn't show *Pseudomalmea diclina* and *Klarobelia inundata* as successive sister species, and relationships among the non-*Oxandra* species are largely unresolved (Figure 4). The ancestor of *Ephedranthus guianensis* and *Ephedranthus amazonicus* is very likely to be androdioecious, which is evident as all *Ephedranthus* species are androdioecious (Lopes and Mello-Silva 2019).

All species in *Oxandra* Clade 1 have an impressed or flat primary vein, this seems to be a synapomorphy for this clade. All species in *Oxandra* Clade 2 have a raised primary vein, except for the clade containing *O. bolivarensis* and both *O. espintana* entries, which have an impressed or flat primary vein. The ancestral state for the clade including all species except the outgroup *Malmea*, is different between the plastid and the nuclear phylogeny: according to the plastid phylogeny it is most likely that the ancestor had a raised primary vein, while the nuclear phylogeny shows an impressed or flat primary vein to be most likely.

Three different states were considered for the rumination of the seed: lamellate, spiniform or peg-shaped. Only in *Oxandra* there were polymorphisms in this character, whereas all other species showed no intraspecific variation. Spiniform ruminations occur in every *Oxandra* species part of Clade 1 and Clade 2, but not in *O. sessiliflora* and *O. unibracteata*. If we consider the nuclear phylogeny, spiniform ruminations seem to be a synapomorphy for the combined *Oxandra* clades, as it occurs in both clades but not in the any species of the (poorly resolved) sister group. This is not the case when looking at the plastid phylogeny, where spiniform ruminations seems to have originated independently in each *Oxandra* clade. *Oxandra espintana* is the only species that has all three types of ruminations.

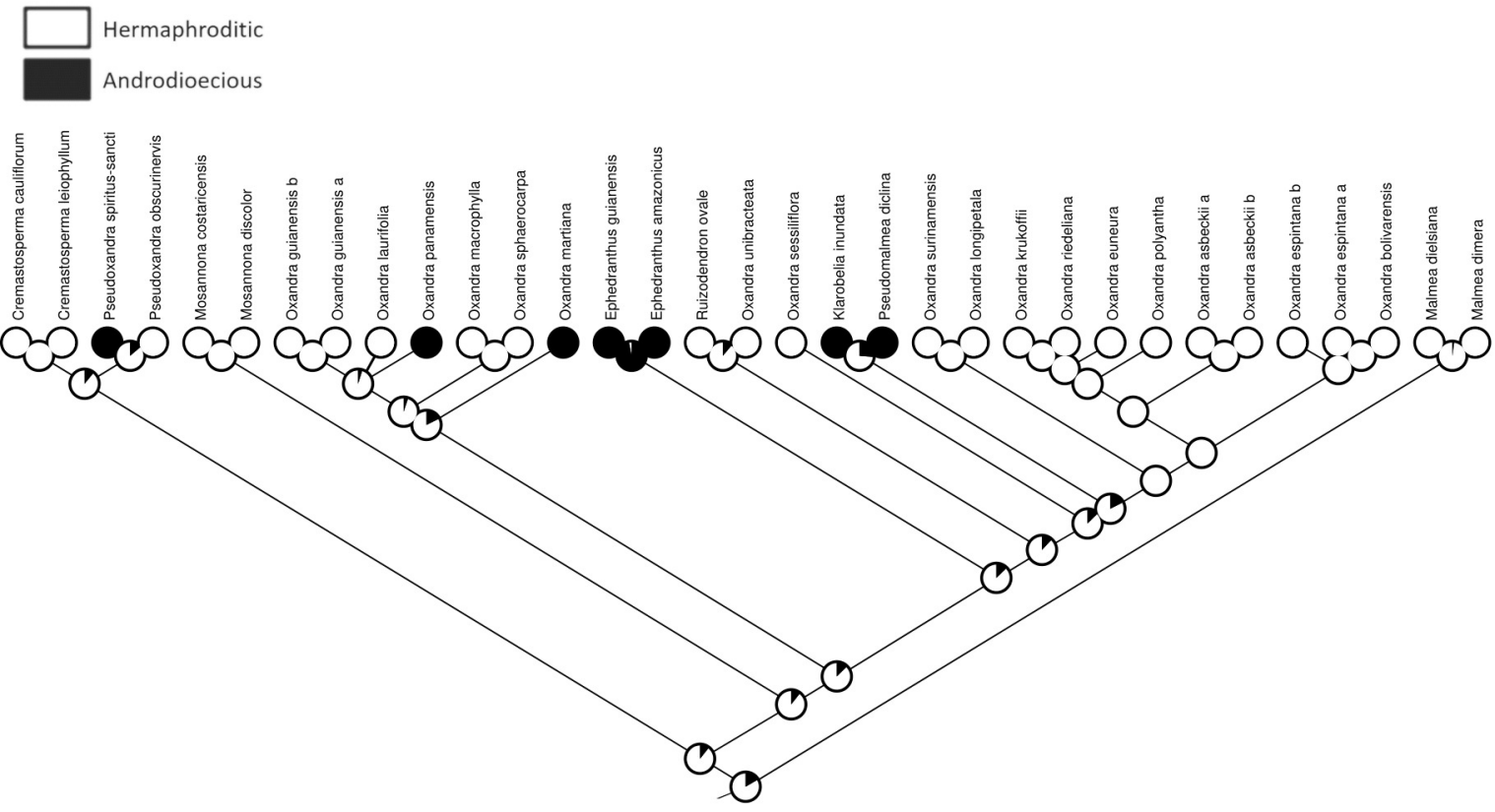

Figure 3: Ancestral state reconstruction of mating system based on plastid phylogeny, using maximum likelihood.

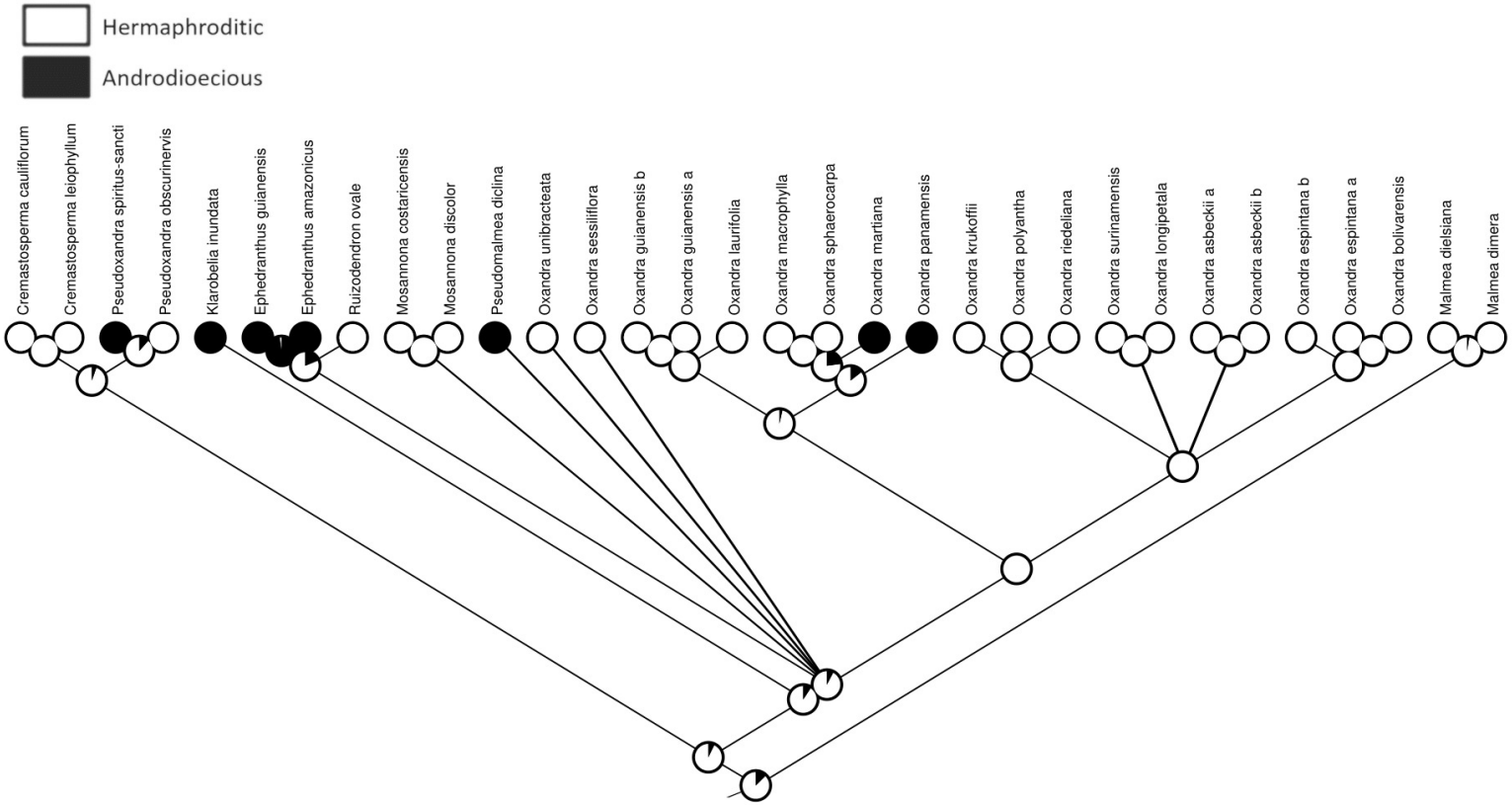

Figure 4: Ancestral state reconstruction of mating system based on nuclear phylogeny, using maximum likelihood.

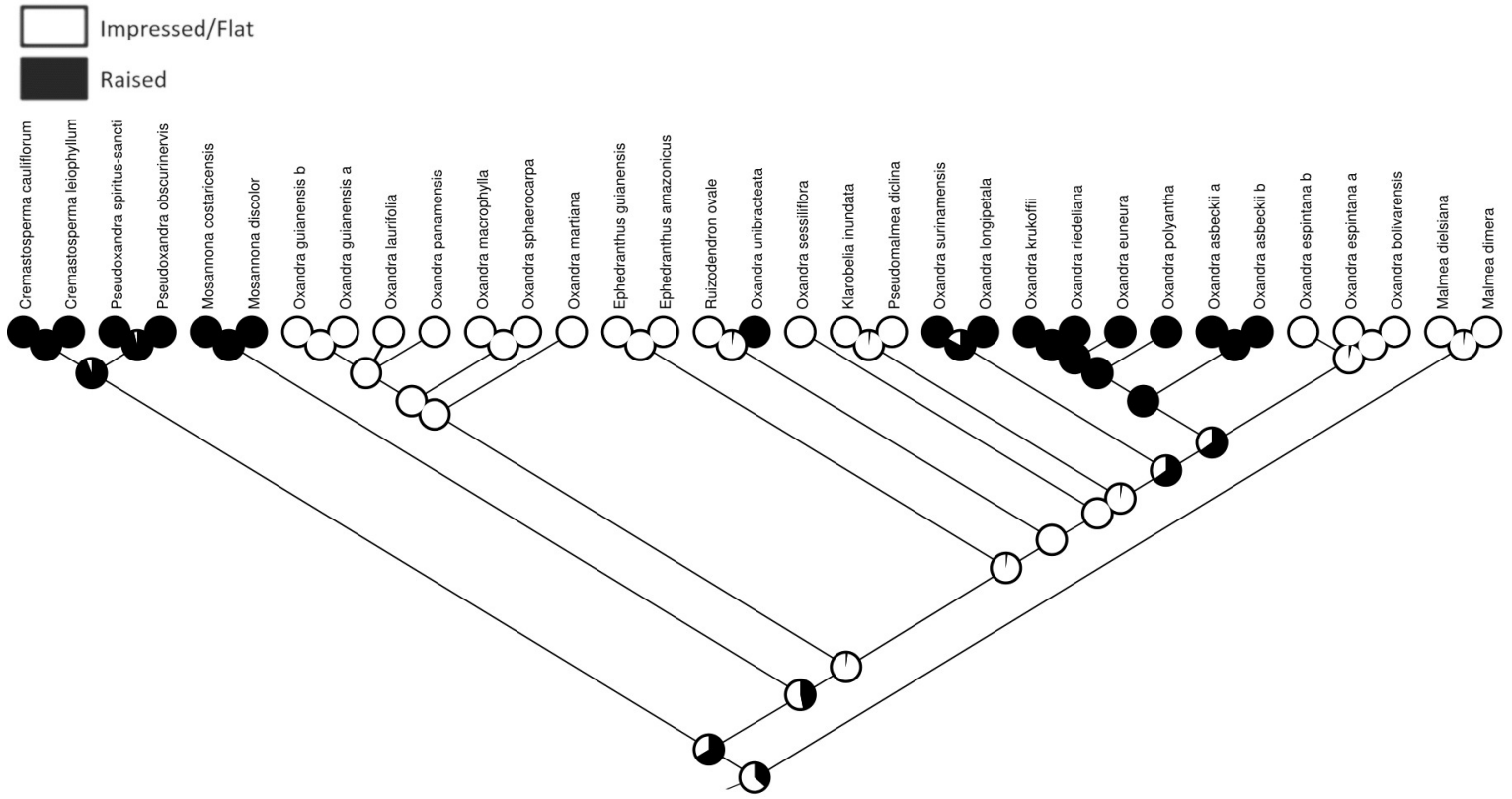

Figure 5: Ancestral state reconstruction of the primary vein placement based on plastid phylogeny, using maximum likelihood.

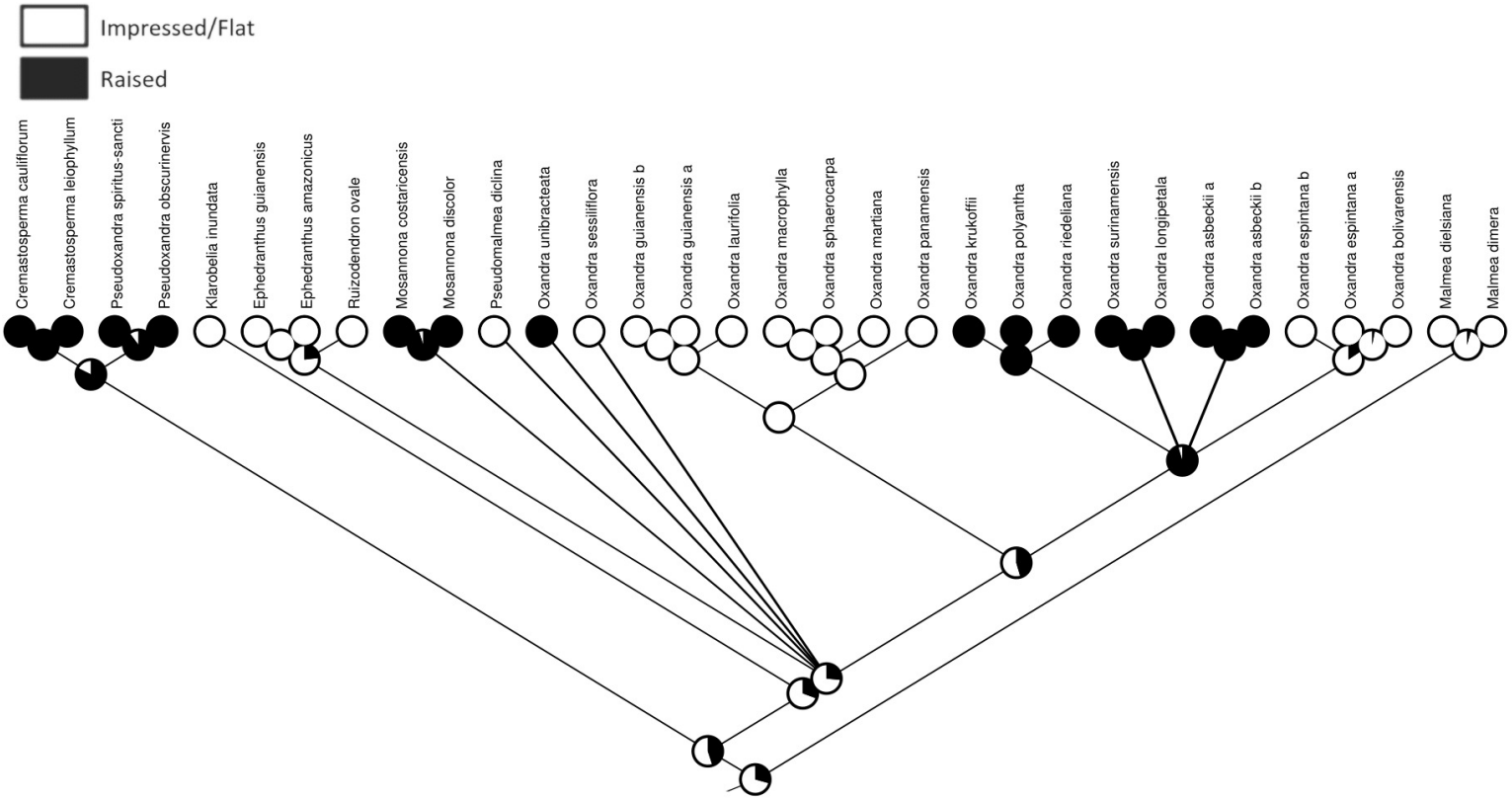

Figure 6: Ancestral state reconstruction of primary vein placement based on nuclear phylogeny, using maximum likelihood.

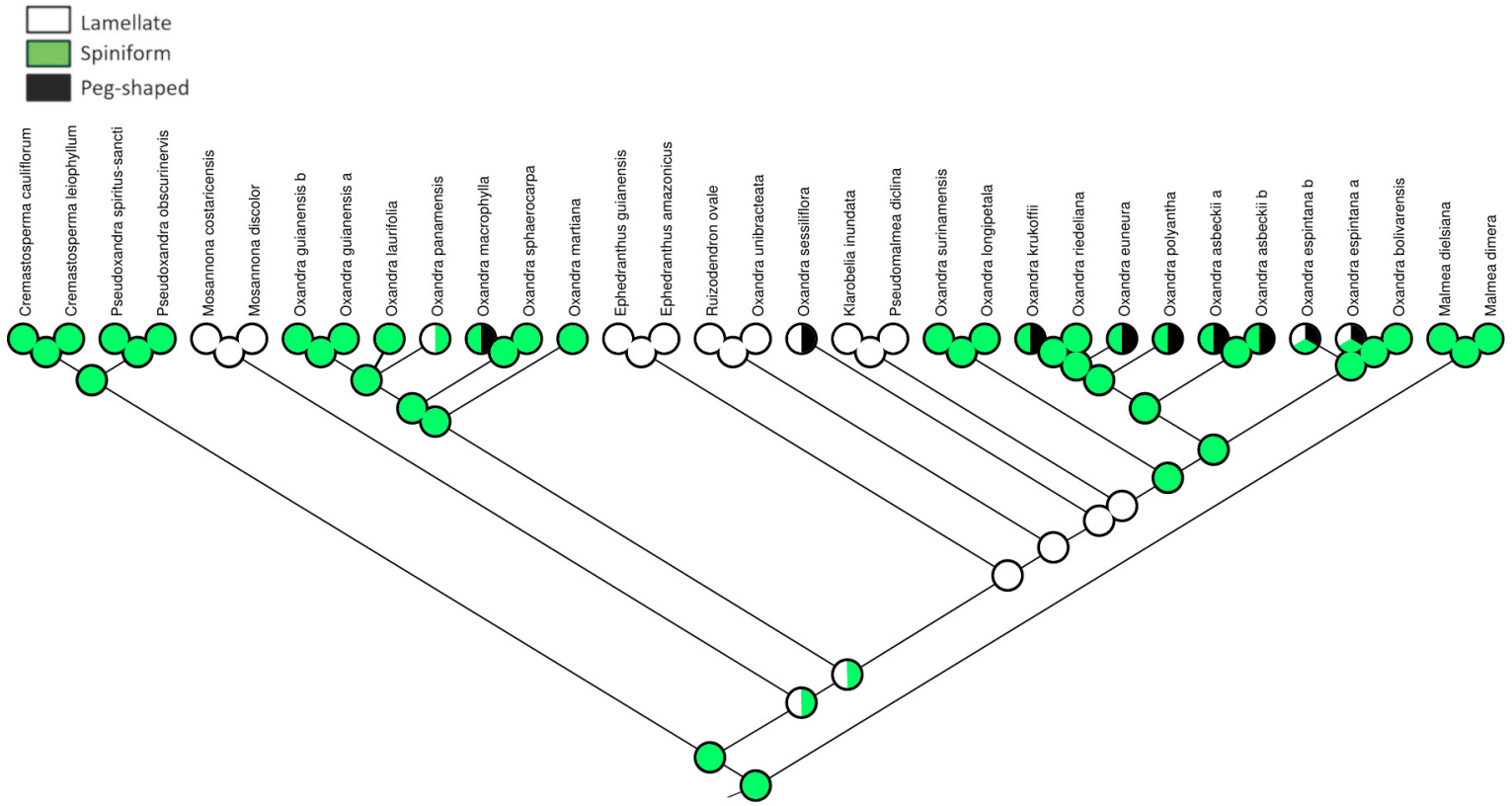

Figure 7: Ancestral state reconstruction of seed rumination based on plastid phylogeny, using maximum parsimony.

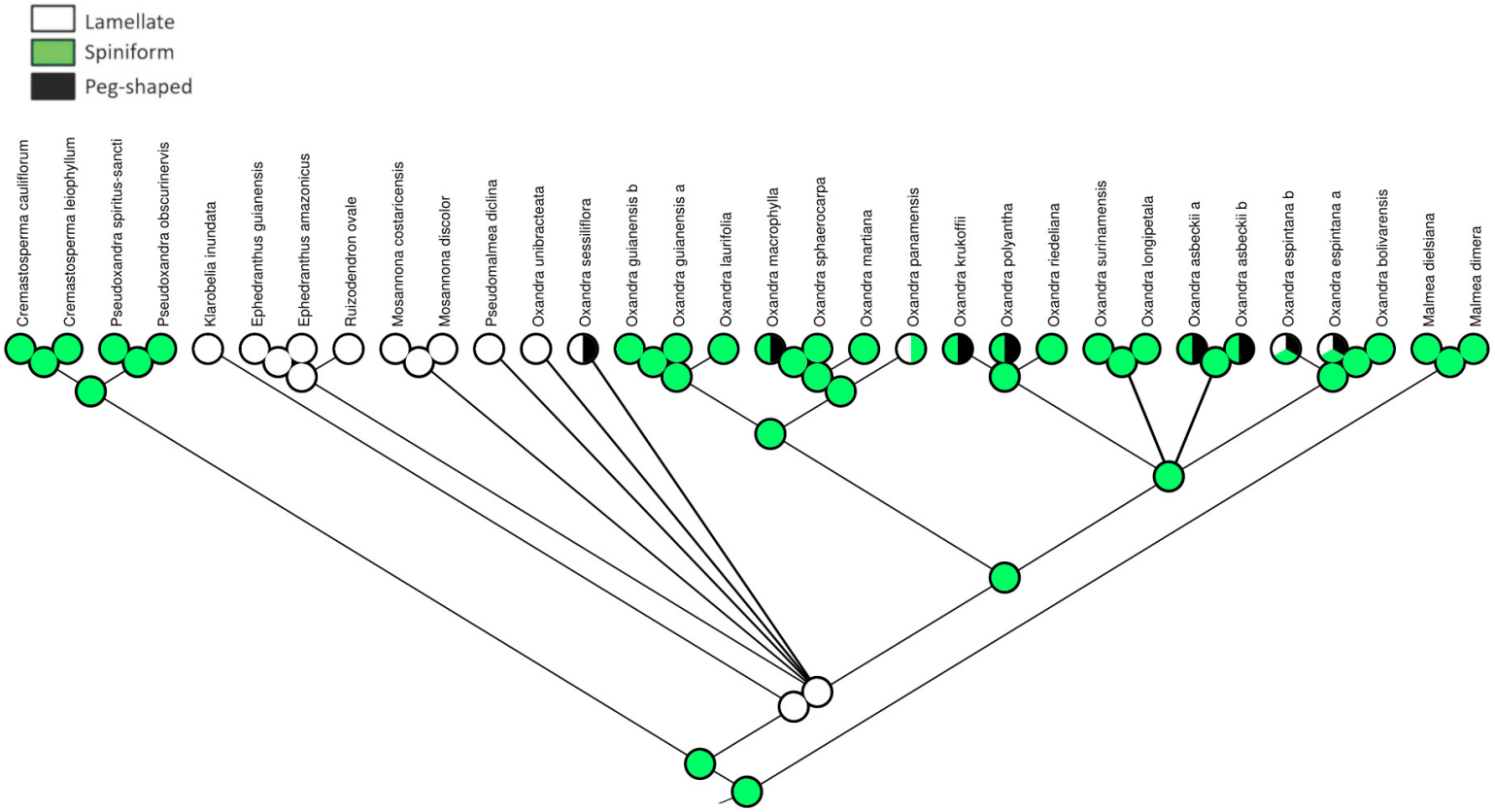

Figure 8: Ancestral state reconstruction of seed rumination based on nuclear phylogeny, using maximum parsimony.

Discussion

Phylogenetic incongruence

The results found by earlier research was also recovered in this study: there is a clear incongruence between the nuclear and the plastid phylogeny. While the species make-up of the two *Oxandra* clades are identical in both phylogenetic trees, their placement in the tree is not. Such an incongruence between phylogenies has been found before (e.g. Sun et al. 2015; Pelser et al. 2010; Galbany-Casals et al. 2014; de Sousa, Bertrand, and Pfeil 2016). Except for technical difficulties such as contamination or failed assemblies, Wendel and Doyle (1998) suggest the following explanations: hybridization/introgression, incomplete lineage sorting (ILS) and horizontal gene transfer. Horizontal gene transfer of mitochondrial genes seems to be rather widespread (Richardson and Palmer 2007), but chloroplast gene transfer is thought to be rare, although it has been found in Annonaceae (Pirie et al., 2007). Still, chloroplast capture could be an explanation for the pattern seen here and it has been proven to occur, both via phylogenetic analyses (Cristina Acosta & Premoli, 2010; Liu et al., 2020) and experimentally (Stegemann et al., 2012). Incomplete lineage sorting describes a phenomenon whereby one or more genes have a different genealogy compared to the species phylogeny. A phylogeny based on these 'incorrectly' sorted genes will thus differ from the true species phylogeny, which would be returned by most of the genes. This phenomenon occurs mostly between closely related species, as the more distantly related two species are, the higher the chance becomes that all genes sort accordingly. Looking at the plastid phylogeny, both

Oxandra clades are split relatively far apart, with some other species between them. It seems rather unlikely that the incorrect sorting was retained over all those genera. Moreover, incomplete lineage sorting is expected to occur more often during fast speciation events. Not much is known about the time at which *Oxandra* originated, but for some closely-related genera such as *Cremastosperma, Klarobelia* and *Mosannona*, Pirie et al. (2006) showed that each genera's most recent common ancestor has originated in roughly the same time window. However, the error margins are too big to exclude separate origins. Overall, it seems like incomplete lineage sorting is a rather unlikely explanation for the pattern found. Lastly, hybridization can also cause phylogenetic incongruence. A single hybridization event would give two distinct phylogenies, that are consistent over gene compartment: if single nuclear copy genes are sampled, they should all give the same consistent pattern. This is different from the more chaotic pattern that would appear due to incomplete lineage sorting.

It has to be noted that preliminary research shows that a phylogeny based on mitochondrial markers shows the same pattern as the nuclear phylogeny. This is somewhat unexpected, as both the mitochondria and the chloroplasts in most plants are inherited maternally, so one would think that they would show the same pattern in case of a hybridization event. However, it has been shown that the progeny of a hybrid has a higher chance to show 'paternal leakage', when an organellar genome is inherited paternally instead of maternally (Xu, 2005). This might give an explanation of the different topology of the mitochondrial and chloroplast phylogeny, in case a hybridization event happened.

Another point to touch upon is the placement of *O. bolivarensis* and both entries of *O. espintana*. *O. bolivarensis* is in both phylogenies nested in *O. espintana*, which would make this species polyphyletic. Junikka et al. (2016) defined *O. espintana* as a combination of *O. espintana* and the former species *O. nitida*. This causes *O. espintana* to have a peculiar distribution, occurring in the Amazonian rain forest, but also in the Atlantic coastal forests in Brazil (Junikka et al. 2016, Map 2). Indeed, the accession closest related to *O. bolivarensis* was sampled in Venezuela while the other entry was sampled close to the Brazilian coast. Taking into account the distinct geographic pattern and the phylogenetic result of this study, it might be interesting to reconsider the broad *O. espintana* species concept in order to obtain monophyletic species.

The overall plastid phylogeny is consistent with the phylogeny obtained by Lopes et al. (2018, Fig 2), where the same pattern of two distinct *Oxandra* clades, separated by a clade containing *Klarobelia* and *Pseudomalmea* (and *Pseudephedranthus* in the study of Lopes et al., 2018) and a branch containing *Ruizodendron* can already be seen. Due to unresolved relationships in the nuclear phylogeny, it is difficult to compare this phylogeny with the plastid one, but (next to the different placing of the *Oxandra* clades) *Klarobelia* takes a more basal position in the nuclear phylogeny compared to its placement in the plastid phylogeny. Also, *Ephedranthus* and *Ruizodendron* are considered as one clade in the nuclear phylogeny, albeit with very low support values. Overall this study confirms the relationships as found by Lopes et al. (2018), but also clearly shows that it is not enough to base a phylogeny only on one part of the plant genome such as the chloroplast. Other genetic entities such as the mitochondrion or single

copy nuclear genes might tell a very different story and shed a new light on the relationships in a clade.

Ancestral state reconstruction

Both androdioecious *Oxandra* species sampled in this study belonged to Clade 1. Unfortunately, it is not possible to draw conclusions about the occurrence of androdioecy in *Oxandra,* as there are no data of the two other species (*O. maya* and *O. mediocris*). Still, according to the maximum likelihood reconstructions, it seems likely that androdioecy originated at least twice in *Oxandra*, both in the plastid and the nuclear phylogeny. Hermaphroditism is the ancestral state in the tribe Malmeeae (Lopes et al. 2018), so all occurrences must have originated from a hermaphroditic ancestor. This is different from what is considered to be the most frequent pattern, namely androdioecy evolving from a dioecious ancestor (Pannell, 2002). If originating from a hermaphroditic ancestor, theory predicts that the androdioecious morph has to produce twice the offspring of the hermaphroditic morph in order to persist in a population (Charlesworth, 1984). However, it is currently not known whether the morphologically androdioecious *Oxandra* species are also functionally androdioecious. A defect in the male function of a hermaphroditic plant would make the species functionally dioecious. The advantage of being functionally dioecious would be that it prevents inbreeding, while keeping the advantage of the morphological hermaphroditic flowers: sterile pollen-producing staminodes would still give pollen rewards for pollen-consuming pollinators (Saunders, 2010). It has to be noted though that the dioecious advantage of avoiding inbreeding might be overestimated here, as Annonaceae have developed different mechanisms to avoid inbreeding (Pang & Saunders, 2014). True androdioecy has been confirmed for *Pseudoxandra spiritus-sancti* (Lopes et al. 2018), but not for any *Oxandra* species. Without knowing this it is not possible to assess the selective advantage of androdioecy in *Oxandra*.

The primary vein placement seems to be consistently impressed/flat in *Oxandra* clade 1. Clade 2 has mostly raised veins except for the clade with *O. espintana* and *O. bolivarensis*. Primary vein placement seems to be a stable character in Malmeeae: all *Ephedranthus, Klarobelia, Malmea* and *Pseudomalmea* species have a flat/impressed primary vein (Chatrou 1998; Lopes and Mello-Silva 2019), while *Pseudoxandra* and *Pseudephedranthus* have a raised primary vein (R. H. J. Erkens et al., 2017; Maas & Westra, 2003). *Cremastosperma* has also a raised vein, which is grooved, a synapomorphy for the genus. It is interesting that a character that seemingly has so little impact on the life history of a plant, is so consistent within genera or clades. One could speculate that the gene(s) coding for primary vein placement might be located close to genes that are selected upon during speciation, making this character so consistent.

Seed rumination was the third character looked at. Contrary to the other characters, there were species with intraspecific variation in the seed rumination type. Interestingly, only in *Oxandra* there are polymorphic species, all other genera sampled here have even consistent ruminations at genus level (Chatrou 1998; Maas and Westra 2003; Lopes and Mello-Silva 2019; Erkens et al. 2017; Pirie, Chatrou, and Maas 2018). Peg-shaped ruminations only occur in

Oxandra, not only in both clades but also in *O. sessiliflora*. Maybe if more subdivisions are made, such as different types of lamellate ruminations, a different pattern would appear.

Overall, the three characters included in this study were much more variable in *Oxandra* then in other genera included in this study. The primary vein placement and seed rumination are synapomorphies for other genera, while differing within *Oxandra* or even within *Oxandra* species (for seed rumination). Especially if we also take into account the phylogenetic incongruence and the fact that at least two species don't belong to either clade, there are signs that *Oxandra* might need some taxonomical changes.

Conclusion

The genus *Oxandra* is divided into two main clades that have a consistent species composition in the plastid and in the nuclear phylogeny. However, their placement in the tribe Malmeeae is different: the nuclear phylogeny renders the two clades as sister clades, while the plastid phylogeny puts them separated from each other. It would be very interesting to see what this pattern looks like with all *Oxandra* species included. Making a phylogeny based on single copy nuclear genes or the mitochondrial genome would also be interesting.

Androdioecy has originated at least two times in *Oxandra*. However, it is currently not known if there is a defect in the male function of the bisexual flowers, which would make the species functionally dioecious. If such a defect would be discovered, it would be the first dioecious species in the Malmeeae. Without knowing if species are truly androdioecious, it is impossible to think about what the evolutionary advantages of such a rare mating system could be.

Summary

Androdioecy is the occurrence of male and bisexual individuals within the same species. It is considered to be very rare within Angiosperms, having originated only a few times independently. In *Oxandra* (Annonaceae), a genus of mostly trees and some shrubs occurring in South America, four species are androdioecious. As androdioecy is so rare, it would be interesting to study the evolution of this character in *Oxandra* and closely related genera. In order to make assumptions about the evolution of androdioecy, a robust phylogeny is needed. Previous studies have always found *Oxandra* to be polyphyletic. However, preliminary data showed the relationships to be more nuanced. Two clades containing most *Oxandra* species were recovered, which are sister clades based on a phylogeny with nuclear markers. The plastid phylogeny however places the two clades further apart. This phylogenetic incongruence can have different explanations such as hybridization, incomplete lineage sorting or chloroplast capture. This study tries to make a phylogeny of all *Oxandra* species, and if the incongruence is recovered, we will try to shed some light on the possible explanations. This phylogeny will be used to perform an ancestral state reconstruction of mating system and other relevant characters. Material of *Oxandra* species not yet sequenced

was gathered from herbarium and silica specimens. DNA extraction was performed using CTAB extraction and a library was prepared to perform genome skimming, a technique which sequences the high copy part of the plant genome. Unfortunately, it was not possible to use these data, so analysis continued using a prepared matrix of the rDNA and one of the entire plastome. A phylogeny was obtained using maximum likelihood and Bayesian inference. Ancestral state reconstruction was performed for mating system, primary vein placement and seed rumination, as the last two are important in species identification. The phylogenetic incongruence was recovered with maximum support. The ancestral state reconstruction showed that androdioecy originated at least twice independently in *Oxandra.* An impressed/flat primary vein was a synapomorphy for one of the *Oxandra* clades, while the other clade had mostly raised primary veins. Compared to other genera in the tribe Malmeeae, seed rumination type was very inconsistent in *Oxandra*, as even intraspecific variation occurs. *Oxandra* is also the only genus where peg-shaped ruminations occur.

The different explanations for the phylogenetic incongruence are discussed. It is currently not possible to identify what caused the incongruence. It would be interesting to see what the phylogenies look like with all *Oxandra* species and with different markers such as the mitochondrial genome or single copy nuclear markers. One species, *O. espinatana*, that has been recently merged, is recovered as polyphyletic. Overall, this study confirms the relationships in the tribe Malmeeae found in previous studies. As the androdioecious *Oxandra* species have a hermaphroditic ancestor, theory predicts that the androdioecious individuals must produce at least twice the offspring of a hermaphroditic individual in order to persist. However, hermaphroditic plants might also be defect in their male function, making the species functionally dioecious. Whether this is the case in the *Oxandra* species is not known, but this should be studied. Without this knowledge it is not possible to assess the selective advantages playing a role in the shift of mating systems. The other two characters showed much more variation in *Oxandra* compared to other genera in Malmeeae. This, combined with the phylogenetic incongruence, might call for a reconsideration of the taxonomy of *Oxandra*.

Samenvatting

Androdioecie is een reproductiestrategie waarbij er zowel tweeslachtige als mannelijke individuen voorkomen. Het wordt slechts zelden gevonden in Angiosperma. Vier soorten in *Oxandra* (Annonaceae), een genus in Zuid-Amerika dat bestaat uit bomen en houtige struiken, zijn androdioecieus. Omdat androdioecie slechts zo weinig voorkomt, kan het interessant zijn om de evolutie van dit kenmerk te bestuderen in *Oxandra* en nauwverwante genera. Hiervoor moet men beschikken over een stabiele fylogenie. Voorgaande studies hebben aangetoond dat *Oxandra* polyfyletisch is. Voorlopige data hebben echter aangetoond dat het verhaal complexer is. Volgens de fylogenie gebaseerd op ribosomaal DNA werden twee clades gevonden, waartoe de meeste *Oxandra* soorten behoren en die elkaars nauwste verwanten zijn. De fylogenie gebaseerd op chloroplast DNA daarentegen plaatst de beide clades verder uit elkaar. Deze fylogenetische incongruentie kan verschillende verklaringen hebben, zoals hybridisatie, 'incomplete lineage sorting' of opname van de chloroplast. In deze studie probeert men een zo compleet mogelijke fylogenie te maken van *Oxandra.* Als de incongruentie teruggevonden wordt, worden de mogelijke verklaringen hiervoor besproken.

De verworven fylogenie zal vervolgens gebruikt worden voor een reconstructie van de voorouderlijke reproductiestrategiën en andere relevante kenmerken. Herbariummateriaal en silicastalen werden gebruikt voor nog niet gesequeneerde *Oxandra* soorten. DNA-extractie gebeurde door middel van CTAB-extractie. Voor het sequeneren werd de techniek 'Genome skimming' gebruikt, waarbij de veel voorkomende genen zoals het volledige chloroplast genoom en het ribosomaal DNA gesequeneerd worden. Jammer genoeg was het niet mogelijk om deze data te gebruiken. Daarom werd een voorbereide matrix gebruikt voor de verdere analyses. Een fylogenie werd opgesteld met twee technieken: maximum likelihood en Bayesiaanse statistiek. De voorouderlijke reconstructie werd gedaan voor reproductiestrategie, de ligging van de hoofdnerf en de invaginaties van de zaadhuid. Deze laatste twee kenmerken werden gekozen omdat ze belangrijk zijn voor het identificeren van soorten in de Annonaceae. De fylogenetische incongruentie is volledig statistisch ondersteund. De reconstructie van de reproductiestrategie toont dat androdioecie twee keer is ontstaan in *Oxandra.* Een ingezonken/platte hoofdnerf is een synapomorfie voor één van de clades, in de andere clade bevinden zich vooral soorten met een uitstekende hoofdnerf. Het patroon van de invaginaties van de zaadhuid is consequent in alle genera van het tribus Malmeeae, maar niet in *Oxandra*, waar zelfs intraspecifieke variatie voorkomt. *Oxandra* is ook het enige genus waar pinvormige uitstulpingen voorkomen.

Vervolgens worden de verschillende verklaringen voor de fylogenetische incongruentie besproken. Op basis van dit onderzoek is het echter moeilijk om te weten welke verklaring de juiste is. Het zou interessant zijn om te weten hoe de fylogenieën van het mitochondrion en andere nucleaire genen eruitzien. Er werd aangetoond dat *Oxandra espintana* polyfyletisch is, hoewel de soort nog maar juist aangepast was naar een breder soortbegrip. Over het algemeen werden de verwantschappen, die teruggevonden werden in voorgaande studies, bevestigd. Aangezien ze tweeslachtige voorouders hebben, zouden de androdioecieuze individuen theoretisch gezien twee keer zoveel nakomelingen moeten hebben om hun voortbestaan te garanderen. Het zou echter ook kunnen dat het mannelijk gedeelte van de tweeslachtige individuen defect is, waardoor de soort in essentie tweehuizig wordt. Of dit zo is voor de soorten in *Oxandra*, is niet geweten. Hierdoor is het onmogelijk om een idee te hebben van welke selectiedrukken een rol spelen in de overgang van reproductiestrategie. De andere twee kenmerken waren veel variabeler in *Oxandra* dan in andere genera in de Malmeeae. Als we dit feit combineren met de gevonden fylogenetisch incongruentie, zijn er signalen dat het tijd is voor een taxonomische herziening van *Oxandra*.

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Appendix

Species	Mating system	Primary vein	Ruminations
S1_Oxandra_espintana	0	0	1+2+0
S2_Oxandra_bolivarensis	0	0	1
S3_Oxandra_sphaerocarpa	0	0	1
S4_Oxandra_unibracteata	0	1	0
S5_Oxandra_espintana	0	0	1+2+0
S6_Oxandra_panamensis	1	0	0 + 1
S7_Oxandra_guianensis	0	0	1
S8_Oxandra_riedeliana	0	1	1
S9_Oxandra_sessiliflora	0	0	0 + 2
S10_Oxandra_krukoffii	0	1	1 + 2
S11_Oxandra_surinamensis	0	1	1
S12_Oxandra_asbeckii	0	1	1 + 2
S13_Oxandra_asbeckii	0	1	1 + 2
S14_Oxandra_euneura	0	1	1 + 2
S15_Oxandra_guianensis	0	0	1
S16_Oxandra_laurifolia	0	0	1
S17_Oxandra_longipetala	0	1	1
S18_Oxandra_macrophylla	0	0	1 + 2
S19_Oxandra_martiana	1	0	1
S20_Oxandra_polyantha	0	1	1 + 2
S21_Cremastosperma_cauliflorum	0	1	1
S22_Cremastosperma_leiophyllum	0	1	1
S23_Ephedranthus_guianensis	1	0	0
S24_Ephedranthus_amazonicus	1	0	0
S25_Klarobelia_inundata	1	0	0
S26_Malmea_dielsiana	0	0	1
S27_Malmea_dimera	0	0	1
S28_Mosannona_costaricensis	0	1	0
S29_Mosannona_discolor	0	1	0
S30_Pseudoxandra_spiritus-sancti	1	1	1
S33_Pseudoxandra_obscurinervis	0	1	1
S34_Pseudomalmea_diclina	1	0	0
S35_Ruizodendron_ovale	0	0	0

Table 3: Character matrix used in ancestral state reconstruction.

	0	1	2
Mating system	Hermaphroditic	Androdioecious	
Primary vein	impressed/flat	raised	
Ruminations	Lamellate	spiniform	peg-shaped

Table 4: Clarification for the characters used in the ancestral state reconstruction.