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Molecular Cytogenetics and Phylogenetic Modeling to Study Chromosome Evolution in the Araceae and Sex Chromosomes in the Cucurbitaceae

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As minhas famílias, a brasileira e a alemã, e aos meus amigos

To my Brazilian and German families and to my friends

PREFACE

STATUTORY DECLARATION

Erklärung

Diese Dissertation wurde im Sinne von §12 der Promotionsordnung von Prof. Dr. Susanne S. Renner betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

____

Aretuza Sousa dos Santos
(Unterschrift)

1. Gutachter: Prof. Dr. Susanne S. Renner

2. Gutachter: Prof. Dr. Günther Heubl

Note

In this thesis, I present the results from my doctoral research, carried out in Munich between September 2010 and February 2014 under the guidance of Professor Susanne Renner. The results from my thesis have contributed to four manuscripts presented in Chapters 2 to 5 of which three are published (Chapter 2 to 4) and one is in review (Chapter 5). For the paper presented in Chapter 2, I reviewed all chromosome numbers published for species of Araceae (Appendix and supplementary data Table S1) and contributed to the writing of the discussion, while for the other three papers (Chapters 3 to 5), I generated all data and conducted all analyses myself. Writing and discussion involved collaboration with Susanne Renner. I also presented the seminars and posters listed below.

______________________	______________________
Aretuza Sousa (Unterschrift)	Prof. Susanne S. Renner (Unterschrift)

Seminars

SOUSA, A. February 2012. Using fluorescence *in situ* hybridization (FISH) to infer the mechanisms responsible for chromosome number changes in the Araceae. *Systematic Botany and Mycology Seminar Series – LMU*, Munich, Germany.

SOUSA, A. October 4-6 2013. From "*x*" to phylogenetics: Inference of chromosome number evolution in 2013. *Summer School in Plant Evolution and Systematics*, Bad Feilnbach, Germany.

Posters

SOUSA, A., CUSIMANO, N. and S.S. RENNER. April 2012. Testing strong predictions about the direction of chromosome evolution in *Typhonium*. 11th Gatersleben Research Conference on Chromosome Biology, Genome Evolution and Speciation. 23-24 April 2012, Gatersleben, Germany.

SOUSA, A., HOLSTEIN, N. and S.S. RENNER. April 2012. *Coccinia grandis*, the plant with the largest known Y chromosome: Characterizing its male and female karyotypes by FISH. 23-24 April 2012, Gatersleben, Germany.

List of publications

CUSIMANO, N., SOUSA, A. and S.S. RENNER. 2012. Maximum likelihood inference implies a high, not a low, ancestral haploid chromosome number in the Araceae, with a critique of the bias introduced by "*x*". *Annals of Botany* 109: 681 – 692.

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SOUSA, A. and S.S. RENNER. Descending dysploidy, unusually large interstitial telomere bands, and chromosome evolution in the monocot family Araceae (in review)

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SUMMARY

This study involved the combination of molecular-cytogenetic data and phylogenetic approaches to infer pathways by which chromosome numbers and sizes may have changed during the course of evolution. The two systems for which I generated new data are the monocot plant family Araceae and *Coccinia*, a genus of Cucurbitaceae. Araceae have about 3800 species in 118 genera, and chromosome numbers range from $2n = 168$ to $2n = 8$, the latter the lowest number so far and newly reported in my study. The small genus *Coccinia* includes *C. grandis*, with the largest known Y chromosome in plants, as documented in my work. The thesis comprises four published or submitted papers.

The first paper reports the result of phylogenetic modeling of chromosome number change along a phylogeny for the Araceae with 113 genera represented. I used a maximum likelihood approach to find the most likely combination of events explaining today's chromosome numbers in the 113 genera. The permitted events were chromosome gains (i.e. breaks), losses (i.e. fusions), doubling (polyploidization), or fusion of gametes with different ploidy. The best-fitting model inferred an ancestral haploid number of 16 or 18, higher than previously suggested numbers, a large role for chromosome fusion, and a limited role of polyploidization. The sparse taxon sampling and deep age (at least 120 Ma) of the events near the root of Araceae caution against placing too much weight on "ancestral" numbers, but inferred events in more closely related species can be tested with cytogenetic methods, which I did in two further studies (papers 2 and 3).

I selected *Typhonium*, with 50-60 species, a range of $2n = 8$ to $2n = 65$ chromosomes. The family-wide study had suggested a reduction from $a = 14$ to 13 by fusion in *Typhonium*, but had included relatively few of its species. I built a phylogeny that included 96 species and subspecies sequenced for a nuclear and two chloroplast markers, and then selected 10 species with $2n = 8$ to 24 on which to perform fluorescence *in situ* hybridization (FISH) with three chromosomal probes (5S rDNA, 45S rDNA, and *Arabidopsis*-like telomeres; paper 2). The results supported chromosome fusion in two species where I found interstitially located telomere repeats (ITRs), which can be a signal of end-to-end fusions, and polyploidization in one species where I found multiple rDNA sites. I then extended my cytological work to other lineages of Araceae, selecting 14 species from 11 genera in key positions in the family phylogeny, which I enlarged to 174 species, adding new chromosome counts and FISH data for 14 species with $2n = 14$ to $2n = 60$ (paper 3). With the new data, I confirmed descending

dysploidy as common in the Araceae, and I found no correlation between the number of rDNA sites and ploidy level (which would have pointed to recent polyploidy). I detected ITRs in three further species, all with $2n = 30$. I also discovered gymnosperms-like massive repeat amplification in *Anthurium*. Similar ITRs are only known from *Pinus* species.

Paper 4 presents molecular-cytogenetic data for *Coccinia grandis*, one of a handful of angiosperms with heteromorphic sex chromosomes. The male/female C-value difference in this species is 0.09 pg or 10% of the total genome. My FISH and GISH results revealed that the Y chromosome is heterochromatic, similar to the Y chromosomes of *Rumex acetosa*, but different from the euchromatic Y chromosome of *Silene latifolia*; it is more than 2x larger than the largest other chromosome in the genome, making *C. grandis* an ideal system for sequencing and studying the molecular steps of sex chromosome differentiation in plants.

Chapter 1

General Introduction

The investigation of chromosome numbers has a long tradition in plant systematic research. Since Carl Wilhelm von Nägeli first identified chromosomes in pollen mother cells in 1842, angiosperm chromosome numbers have been published that range from *n* = 2 (*Haplopappus gracilis*: Singh and Harvey, 1975; Yonezawa, 1981; *Zingeria biebersteiniana*: Bennett et al., 1995) to *n* = 250 (*Strasburgeria robusta*: Oginuma et al., 2006) and *n* = ~ 320 (*Sedum suaveolens*: Uhl, 1978). The variation is even higher in ferns, where it ranges from *n* = 9 to *n* = ca. 720 (references in Leitch and Leitch, 2012). Counting and studying chromosomes became popular at the beginning of the 20th century, as the initially independent fields of genetics and cytogenetics developed, focusing on grasshoppers and *Drosophila* on the animal side, and *Bryonia* and a few other "systems" on the plant side (Correns, 1903; Rubin and Lewis, 2000; Crow and Crow, 2002). The word "gene" was coined in 1905. Today, abundant data from light microscopy have made clear that chromosome numbers can vary among closely related species and that single species can have different numbers even in the same population. For example, the common European species *Cardamine pratensis* can have 2*n* = 16, 17, 18, 20, 24, 28, 30, 32, 34, 38, 44, 46, 48, 56, 60, 64, 80, 88, 90: Index to Plant Chromosome Numbers: IPCN, www.tropicos.org/Project/IPCN). Bennett et al. (1995) estimated that perhaps 25% of the angiosperms have had their chromosomes counted, and it is clear that numbers have increased and decreased during the course of evolution, although the mechanisms underlying the changes remain poorly understood.

A similar range of chromosome numbers exists in animals. For example in ants, the chromosome number varies from *n* = 1 in *Myrmecia pilosula* ♂ to *n* = 47 in *Prionomyrmex macrops* (Crosland and Crozier, 1985; Imai et al., 2002). *Myrmecia pilosula*, originally described as one species, was revealed to include several distinct sibling species by the observation of multiple diploid chromosome numbers of 2*n* = 9, 10, 16, 24, 30, 31, and 32 (Crosland and Crozier, 1985). Perhaps the most spectacular case of number variation is that of the muntjacs. The Indian muntjac, *Muntiacus muntjak vaginalis*, has a karyotype of 2*n* = 6 in females and 2*n* = 7 in males, while the Asia muntjac, *M. reevesi*, has 2*n* = 46 (Yang et al., 1997). So far, the highest chromosome number reported for any animal comes from the fishes *Acipenser baerii* with 2*n* = ~368 and *A. brevirostrum* had 2*n* = 372 (Havelka et al., 2014).

Features of a karyotype, such as chromosome number, morphology, and symmetry, can be used along with morphological traits to diagnose a species. These features are not

influenced by external conditions or age and therefore are reliable markers for taxonomic and molecular studies. In plants, it became common practice to propose a so-called basic (or base) number, x, by calculating the smallest common factor of series of haploid chromosome numbers (n) for entire groups (Sansome and Philp, 1932). This concept was never appreciated among zoologists, and appears not to have been used for any animal group; at least I have been unable to find an example.

There are several problems with the "basic number approach." First, the reliability of the inferred number depends on the sampling density, that is, the percentage of individuals and species in a group for which there are counts. Second, botanists commonly take the basic number as the ancestral number in the respective group, yet the approach does not incorporate phylogenetic relationships, and obviously was developed before the availability of morphological or molecular phylogenetic trees and before "tree thinking" took hold in the biological sciences in the 1980s.

The application of x in plant systematics is well illustrated in a masterful review of chromosomal research by Raven (1975). Raven tried to bring new cytological data into a phylogenetic context. The available classification systems at the time, such as those of Cronquist (1968), Thorne (1968), or Takhtajan (1969), were still in the tradition of idiosyncratic groupings that could not be reproduced by scientists other than the author because they were not based on explicit data matrices as became common practice following the Hennigian revolution (Hennig, 1966). Peter Raven reviewed angiosperm chromosome numbers published at the time and proposed base numbers for each plant order (using the orders of Cronquist, every one of which has since turned out to be poly- or paraphyletic). Raven (1975: p. 760) also suggested that "the original basic chromosome number in angiosperms seems clearly to have been $x = 7$, characteristic of all major groups of both dicots and monocots except Caryophyllidae, with $x = 9$." As his own data show, however, a chromosome number $n = 7$ only occurs in Annonaceae, a family in Cronquist's order Magnoliales then seen as "primitive" (Raven 1975: p. 728; today, we would replace primitive by "early-diverging"). One has to remember that this was written before sister-group-thinking, and that Cronquist (1968) believed the Magnoliales had retained many traits from an imagined "original" flowering plant. Other families of the Magnoliales, such as Calycanthaceae, Monimiaceae, Lauraceae, and Myristicaceae, have much higher chromosome numbers. In Figure 1, I have plotted the chromosomes numbers mentioned by Raven (1975)

on the APG phylogeny as simplified by Stevens (2001 onwards). The most common haploid chromosome numbers among today's magnoliids are $n = 12$ and 13, numbers also found in many living gymnosperms (Fig. 1), and in *Amborella*, the sister to all other angiosperms (Chamala et al., 2014 and many earlier references therein).

Raven's basic numbers for all angiosperm orders did not go unchallenged (Grant, 1982), but in the context of this *Introduction*, it is important only to illustrate the many difficulties researchers who wanted to infer chromosome evolution or wanted to use chromosome information in plant systematics were experiencing before the availability of (i) phylogenies obtained in a reproducible manner, (ii) molecular data to infer relationships, and (iii) better ways to infer ancestral traits. In the following, I briefly discuss the progress in these three areas since 1975 because it is directly relevant to the approaches used in my doctoral research.

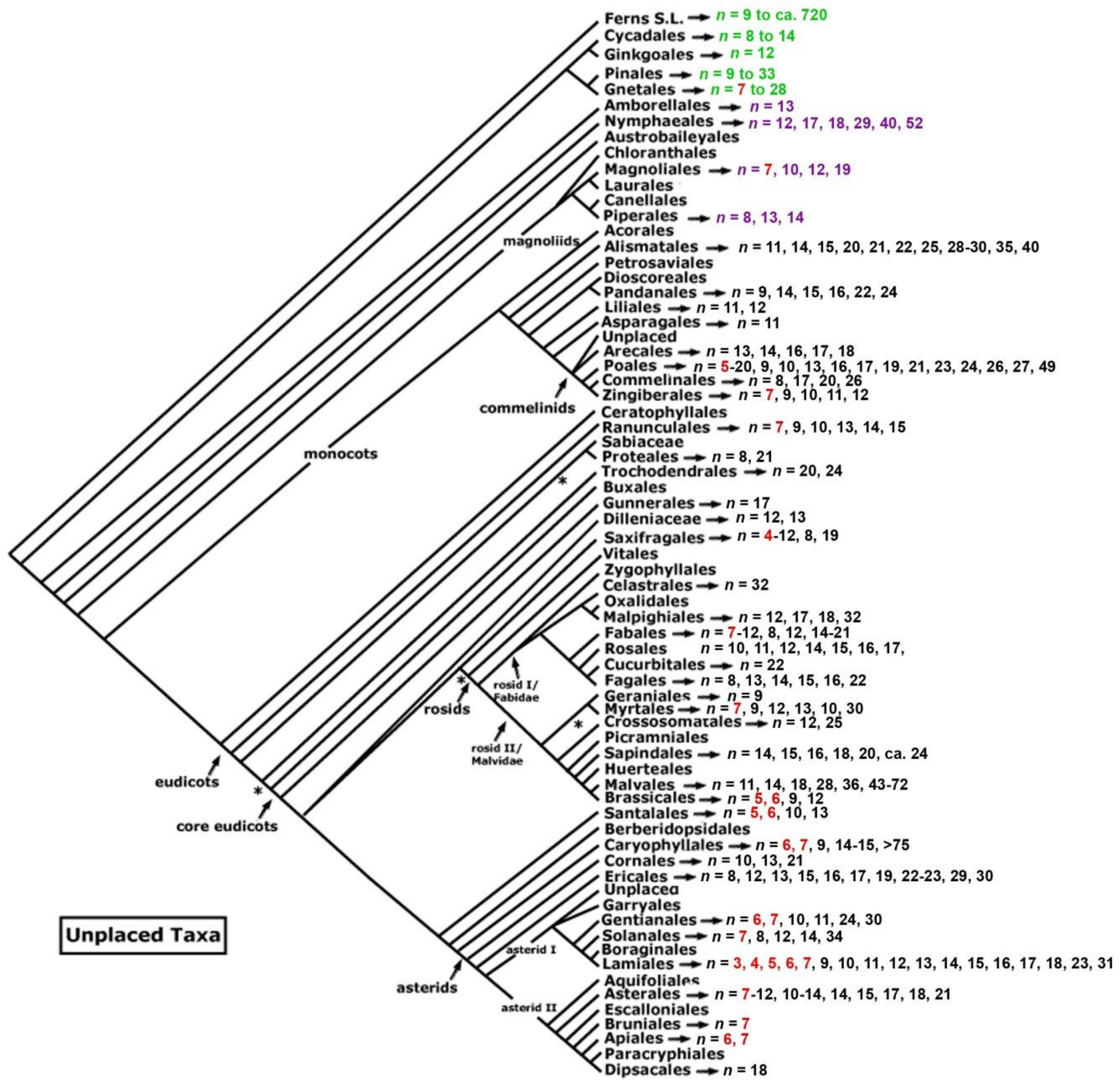


Fig. 1. Mapping of the chromosome numbers provided in Raven (1975) on the current DNA angiosperm phylogeny. Orders and families were searched online to find their current classification (from Cronquist [1968] to APG III). Only haploid or diploid chromosome numbers from Raven (1975) were plotted. Inferred basic numbers (x) are not included. The information for outgroups (green) is from Leitch and Leitch (2012). Numbers of basal angiosperms are shown in purple, others in black. Orders without chromosome numbers reflect the lack of data for these groups until 1975. Numbers in red are the proposed basic numbers (x = 7 or lower) for angiosperms according to Raven.

Progress in tree inference, trait reconstruction, and cytogenetics from 1966 to 2014

The work of Willi Hennig (1966) brought about a paradigm shift in systematic biology by putting forward a method for grouping species and lineages that in principle leads to reproducible and testable results. Although Hennig's approach became quantitative and objective only after the development of computer algorithms by Farris (1970, also Kluge and Farris, 1969), it was Hennig who had the crucial idea of contrasting plesiomorphic with synapomorphic and autapomorphic characters, who realized that synapomorphies could identify sister groups, and who put forward the concept of paraphyly, so essential to his insistence that only monophyla are worth studying and naming (Renner, 2014). The first large DNA phylogenies for plants became available around 1993 (Chase et al., 1993; Steven et al., 2001 onwards). They made it possible to understand the origin of land plants and to clarify the relationships among them. A few examples suffice to illustrate the huge changes in our understanding of plant relationships coming from DNA-based and quantitatively analyzed phylogenetic data matrices. Thus, the work of Olmstead and Palmer (1994), based on cpDNA restriction site data, revealed that tomato, classified by Cronquist (1968) in the genus *Lycopersicum*, is embedded in the genus *Solanum*. Koch and collaborators (1999), based on the analyses of nuclear ribosomal DNA (specifically the Internal Transcribed Spacer regions I and II), revealed that the closest relatives of *Arabidopsis thaliana* with $n = 5$ are species until then placed in the genus *Cardaminopsis* with $n = 8$. This result led to a new circumscription of *Arabidopsis* and *Cardaminopsis* (Koch et al., 1999; Soltis and Soltis, 2000). The work of Qiu and collaborators (1998) revealed that *Nelumbo* is related to *Platanus* and other Platanaceae. And finally the work of Davis et al. (2007) revealed that Rafflesiaceae are embedded in Euphorbiaceae. These few examples show the magnitude of the changes resulting from use of DNA matrices to infer phylogenetic relationships. By now, 2014, the new approaches to tree inference and modeling data have remodeled the thinking of an entire generation of biologists (human generation time is 25 years) regarding plant evolution.

Progress in our understanding of chromosome evolution in *Arabidopsis*, however, not only came from statistical molecular phylogenetics. It also depended on the development of fluorescent-*in-situ*-hybridization or FISH (below, p. 9). The combination of molecular phylogenetics and FISH has shown that the 10 chromosomes of *A. thaliana* result from a

series of complex chromosome rearrangements that can be inferred by comparing the *A. thaliana* chromosomes to the 16 chromosomes of the closest relative *A. lyrata* (Lysak et al., 2006). Whole genome sequencing of representative angiosperms also shows that the ancestor of Brassicaceae or Brassicales (to which *A. thaliana* belongs) was involved in multiple whole-genome duplications, WGD, which must have involved huge increases (followed by decreases) of repetitive DNA and probably also ups and downs in chromosome numbers (Jiao et al., 2011). In combination, these results illustrate that there is no simple rule by which to infer the ancestral chromosome number of huge groups of flowering plants.

Today it is clear that low or high chromosome numbers are neither consistently related to the absence or presence of a WGD nor to a species' ancestral or derived evolutionary status. Using chromosome number to try and infer rates of polyploidization in land plants (as done by Wood et al., 2009) is thus simple-minded and will not yield convincing inferences without additional molecular cytogenetic work (Sousa et al., 2014). Instead, evolutionary changes in chromosome numbers need to be inferred separately from evolutionary change in genome size (a study doing both is Pellicer et al. 2014). The evolution of both types of characters (or traits) can be studied by preparing a data matrix with chromosome numbers or C-values (genome sizes) and then tracing the changes on a DNA-based phylogeny that includes the same species or individuals for which the characters of interest have been coded. Two methods of analysis are available, either parsimony or model-based approaches. Parsimony does not include a model of trait change and therefore cannot make use of the information contained in the genetic branch lengths (branches being the connecting lines in the phylogeny, which in parsimony have no information content, while in maximum likelihood they are proportional to the number of substitutions or can be made proportional to time under a clock model of substitution). This is because parsimony only considers synapomorphies as informative, while maximum likelihood uses information from synapomorphies as well as autapomorphies.

There are many examples of parsimony-based inference of changing chromosome numbers. One such study is that of Soltis et al. (2005) who used a DNA phylogeny of 172 genera from almost as many families to test if Raven's (1975) suggested basic number of x = 7 would hold up in the context of their new phylogeny (very different from Cronquist's [1968] classification). The 172 tips in the tree represented the 13400 genera in 450 families of angiosperms. The resulting basic chromosome number for basal-most angiosperm lineages

was equivocal because many early-diverging lineages have high chromosome numbers (data not shown by Soltis et al., 2005). In an alternative coding approach, in which they modified the empirical numbers for 16 species in their tree to reflect supposed genus-specific ancestral numbers (based on the assumption that these species were paleopolyploids), they "reconstructed" an "original" base number for the angiosperms of 6 and 9, close to Raven's (1975) proposed number of 7. Note that Soltis et al. (2005) coded the sister species to all other angiosperms, *Amborella trichopoda*, as $n = 6$ and 7, even though the empirical number of *A. trichopoda* is $n = 13$.

There is a trend in studies of plant chromosome numbers of seeing polyploidization (the duplication of the set of chromosomes) as the main evolutionary source of chromosome number variation. Indeed, polyploidization is a common event in plants. One of the observations supporting this is the high mean frequency of unreduced gametes (0.56% of gametes, rising 50-fold to 27.52% in hybrids; Leitch and Leitch, 2012). However, the increase or decrease by a single chromosome in a karyotype, called dysploidy, may be equally frequent; no hard data are available yet. Dysploidy has been much less studied than polyploidy, and its numerical contribution to the organization of plant genomes is therefore unknown. In animals, dysploidy is the main source of chromosome number change, specially related to fission-fusion cycles or Robertsonian rearrangements (Imai et al., 2002).

Model-based inference of chromosome evolution, and fluorescence *in situ* hybridization

In the previous section, I have discussed an example of parsimony-based inference of change in chromosome numbers, namely Soltis et al. (2005), which stands for many similar studies. I will now turn to model-based approaches, in which the probability of character change along a branch is proportional to the length of that branch. The first and so far only approach implementing a model-based approach to the study of chromosome number change is that of Mayrose et al. (2010). These workers formulated probabilistic models describing the evolution of chromosome number along a phylogeny, and their software allows the user to apply either maximum-likelihood (ML) or Bayesian inference to the data. The input data consist of a maximum likelihood tree in newick format (for the ML approach) or the maximum clade credibility tree (for the Bayesian approach), also in newick format, a table

with the species and their respective haploid chromosome numbers, and a parameter file specifying file location, maximum and minimum chromosome numbers allowed, and number of simulations for computing a null distribution of the number of changes. The analysis then consists in comparing the fit of eight models to the phylogenies, with the following parameters: polyploidization (chromosome number duplication) with constant rate ρ, demi-duplication (fusion of gametes of different ploidy) with constant rate μ, and dysploidization with either constant or linearly changing rates (ascending: chromosome gain rates λ or $\lambda 1$; descending: chromosome loss rates δ or $\delta 1$). As explained in the previous section, the advantage of the maximum likelihood method compared to the parsimony method is that the latter disregards information contained in phylogenetic branch lengths, which tends to underestimate the number of transition events. The advantage of the Bayesian approach compared to both other approaches is that it provides the statistically best way to calculate the uncertainty in ancestral state probabilities and thereby to obtain confidence limits.

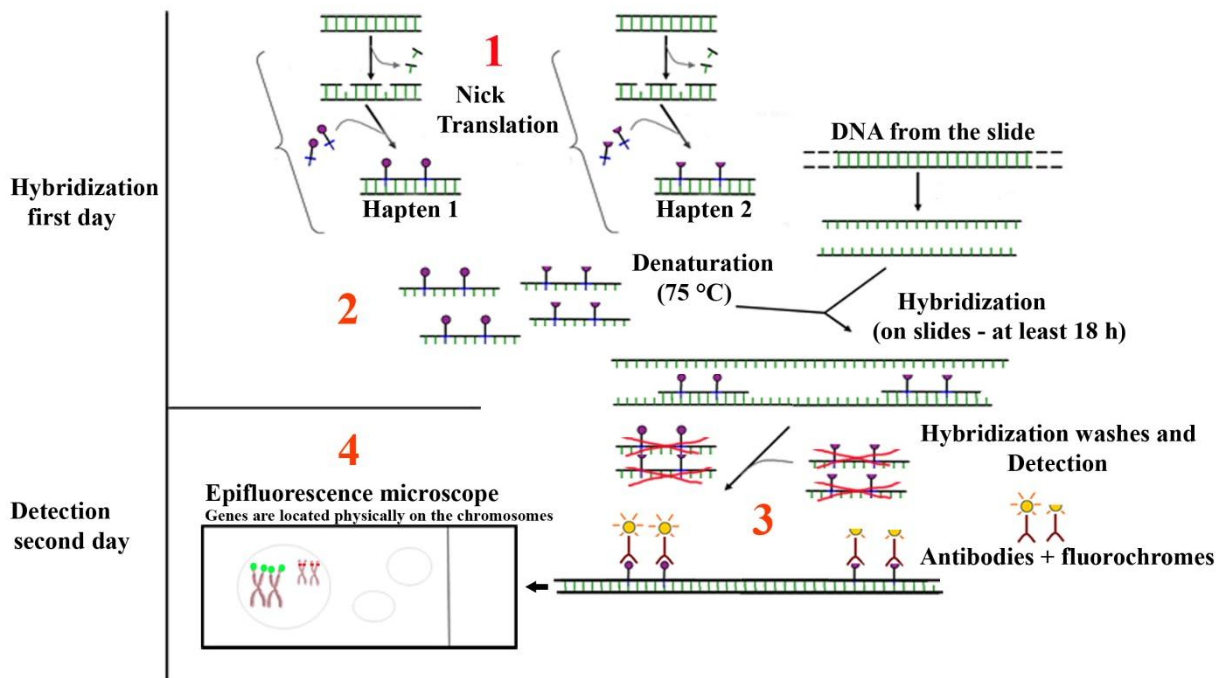
Mayrose et al. (2010) tested the power of their method with artificial data and also with empirical data from *Aristolochia*, *Carex*, *Passiflora*, and *Helianthus*. With the artificial data, they were able to correctly infer (known) chromosome numbers as long as the sampling density was 35% of the total and as long as the root-to-tip distance (the genetic branch lengths, that is, the "length of time") was not longer than 0.76. The approach of Mayrose and collaborators has been applied in clades of Araceae, Iridaceae, Melanthiaceae, Pontederiaceae, Pteridaceae, Portulaceae, Ranunculaceae, and Colchicaceae (Ness et al., 2011; Harpke et al., 2012; Ocampo and Columbus, 2012; Metzgar et al., 2013; Soza et al., 2013; Pellicer et al., 2014; Chacón et al., 2014; Chapters 2 and 3 of this thesis). However, it is difficult to trust the inferred past chromosome numbers without cytogenetic data, especially given the known evolutionary lability of chromosome numbers as illustrated above in *Cardamine pratensis* or *Arabidopsis* (Lysak et al., 2006; Mandáková et al., 2013). Data from genomics and molecular-cytogenetic methods, such as FISH-labelling of chromosomes, remain the best way to search for evidence of evolutionarily recent chromosome number changes (Bowers et al., 2003; Lysak et al., 2006; Peruzzi et al., 2009; Chamala et al., 2014).

I will now give a brief introduction to the FISH approach, which is one of the key methods used in my doctoral research (Chapters 3, 4, and 5). *In situ* hybridization was developed by Gall and Pardue (1969) and John et al. (1969), and initially involved the annealing of radioactive DNA or RNA probes to cytological preparations and their detection

by autoradiography. The major limitations of this method were that it required long exposure time (weeks or months) to detect hybridization sites and the poor resolution of autoradiographs (Rayburn and Gill, 1987). Subsequent modifications resulted in the detection of the hybridization sites after just a few hours and safer handling of the probes, which were no longer radioactive and stable for longer periods (Rayburn and Gill, 1985). Another advantage of the FISH technique is that different DNA probes can be labeled with different haptens (Fig. 2) and detected simultaneously using different fluorochromes (Jiang and Gill, 1994). The fluorescence signals can be captured by special cameras and analyzed with digital imaging systems (Rayburn and Gill, 1985). The principle has also been used to identify parental genomes *in situ* on the chromosomes. In genomic *in situ* hybridization (GISH), the total DNA from the genome of one parent is labeled as a probe, and unlabeled total DNA from the other parent is added in the hybridization mixture (Fig. 3). The preferential hybridization of the labeled genome probe to the chromosomes is taken to indicate the original set of chromosomes, while the other set, blocked by unlabeled DNA and consequently without detectable hybridization signals, is taken to be from the other relative (Brasileiro-Vidal et al., 2005; Markova et al., 2007; Fig. 3).

Fig. 2. (***facing page***) Main steps of fluorescent *in situ* hybridization (FISH). **1.** The labeling of the probe is independent of the slide preparation. The nucleotides of the target DNA are replaced by nucleotides carrying haptens by the nick translation technique. **2.** Next, both the target DNA (probe) and the chromosomal DNA in the slide are denatured by heating, and as the probe consists of small fragments, it hybridizes *in situ* on its native DNA strand faster than the long complementary DNA strand. The hybridization process takes at least 18 hours (steps **1** and **2**). **3.** The experiment is followed by washes to remove the excess of DNA that did not hybridize with the chromosomal DNA and then the detection of the haptens by antibodies associated with fluorochromes. Different DNA regions can be detected at once when they are labeled with different haptens (in the figure hapten 1 and hapten 2). **4.** The final step is the observation of the target chromosomal regions under a fluorescence microscope.

FISH experiment



GISH experiment

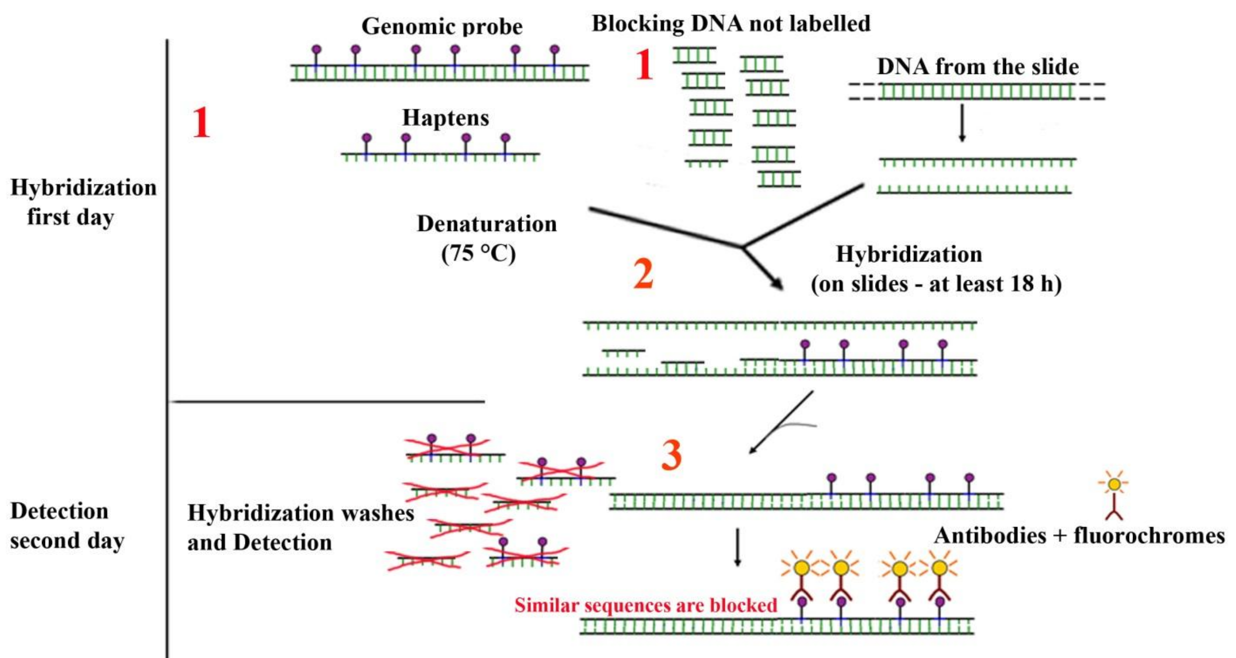


Fig. 3. (***previous page***) Main steps of the genomic *in situ* hybridization (GISH). **1.** Similar to FISH, the labeling of the probe by nick translation, here the genomic DNA from a parent, is independent of the slide preparation. Additionally, the unlabeled DNA from the other parent is added in the hybridization mixture. **2.** Next, the genomic DNA (probe), unlabeled DNA (blocking) and the chromosomal DNA in the slide are denatured by heating. The probe and the blocking DNA will hybridize *in situ* on the chromosomes faster than the long complementary DNA strand. The hybridization process takes at least 18 hours (steps **1** and **2**). **3.** Similar to FISH (Fig. 2). The blocking DNA competes with the probe in regions of DNA similarity, especially when the species are close related. The probe will label the chromosome set of its genomic origin while the blocking DNA does the same, but because it lacks haptens, no hybridization signals will be detected from the blocking DNA.

Changes in chromosomes that can be inferred or tested using fluorescence *in situ* hybridization are structural changes associated mainly with primary (insertions, deletions, duplications, reciprocal translocations, and sequence amplification) or secondary (replication slipping) chromosome rearrangements (Schubert, 2007; Guerra, 2008). So far, none of the eudicots (e.g., *Arabiodpsis*, *Papaya*, soybean, poplar, grape) and monocots (e.g., the Poaceae rice, barley, hordeum) that have had their whole genome sequenced and annotated exhibit the deletion of an entire chromosome (Luo et al., 2009; Abrouk et al. 2010).With the sparse data available so far it instead appears that reciprocal translocation between chromosomes is common, with two chromosomes exchanging chromosomal regions simultaneously (Lysak et al. 2006; Schubert and Lysak 2011, see their Fig. 3). Genome comparisons in the grass family also revealed an unsuspected mechanism of chromosome number reduction, namely insertional dysploidy (Luo et al., 2009). In this case, a complete chromosome is inserted in the centromeric region of another chromosome in a single translocation event, followed by the inactivation of one of the centromeres (Srinivasachary et al. 2007; Luo et al. 2009).

Testing model-basal inferences about chromosome evolution with cytogenetic data

Testing ancestral state reconstructions obtained by parsimony or model-based approaches as described above (p. 10) with FISH data can be done in a manner that I developed during my doctoral research (Chapters 3 and 4): An inferred disploidy event (step-

wise chromosome number increase or loss) would be supported by the discovery of interstitial telomere repeats (ITRs). Due to the normal terminal distribution of telomere repeats to protect the chromosomes against fusions and DNA degradation, the detection of interstitial signals may suggest chromosome reduction by fusion. With probes homologous to plant telomeric repeats one can visualize them (Ijdo et al., 1991; Weiss-Schneeweiss et al., 2004). Since several types of events can lead to interstitial telomere signals, a careful consideration of the specific karyotype(s) being analyzed is required, but in principle the distribution of ITRs can suggest chromosome loss by fusion. A second way to test inferred directions of chromosome number change are sister species comparisons focusing on the distribution and number of 5S rDNA and 45S rDNA sites. An increase in rDNA sites associated to the doubling of the chromosome number might indicate a recent duplication event (Ansari et al., 2008; Souza et al., 2010; Weiss-Schneeweiss et al., 2008) or the observation of rDNA sites in different chromosome regions among species might indicate chromosome rearrangements (da Silva et al, 2008; Souza et al., 2009; Chapter 3 of this thesis).

The DNA probes most used in plant FISH studies are *Arabidopsis*-like telomere repeats, 5S and 45S rDNA. They all belong to a class of repetitive DNAs organized in tandem in specific loci on a chromosome. The 45S rDNA was the first repetitive sequence to be cloned and mapped on the chromosome of plants by *in situ* hybridization (Gerlach and Bedbrook, 1979), followed by the 5S rDNA (Gerlach and Dyer, 1980), and the telomere repeat (Richards and Ausubel, 1988). The 45S rDNA repeat unit consist of an external transcribed spacer (ETS), coding regions of the three rRNA and two internal transcribed spacers, ITS1 (between the 18S and 5.8S genes) and the ITS2 (between the 5.8S and 25S), as shown in Fig. 4a. The coding regions with ca. 1.800, 160, and 3.400 bp respectively, are highly conserved among the eukaryotes (Gerlach and Bedbrook, 1979; Unfried and Gruendler, 1990; Pendás et al. 1993; Murray et al. 2002), both in length and in nucleotide sequence, and they are commonly used as molecular markers in plant molecular cytogenetics (Vaio et al., 2005; Ansari et al., 2008; Sousa et al., 2011). In wheat and barley, a 45S rDNA repeat unit is usually 9 kb to 10 kb long (Gupta, 2010), and overall in plants it ranges from 1 to 15 kb (Rogers and Bendich, 1987; Falquet et al., 1997).

The transcription of rDNA gives rise to the nucleolus, observed in cells in interphase and in prophase (Caperta et al., 2002). The nucleolus disappears with the suspension of gene transcription during the cell division (metaphase-telophase), and the loci with the active rRNA

genes, called nucleolar-organizing regions (NOR), can be visualized in metaphase chromosomes as secondary constrictions (Neves et al., 2005). The transcription of 5S rDNA, different from the 45S rDNA, occurs outside the nucleolus (Sastri et al., 1992; Douet and Torment, 2007; Gupta, 2010), and its conserved coding region consists of 120 bp (Fig. 4b) while the non-transcribed spacer (NTS) varies from 100 to 700 pb (Fig. 4b). In general, the 5S and 45S rRNA genes are located in chromosomal loci independent of each other. However, in some organisms, these genes are intercalated in the same repeat unit (Drouin and Moniz de Sá, 1995; Sone et al., 1999; Garcia et al., 2009).

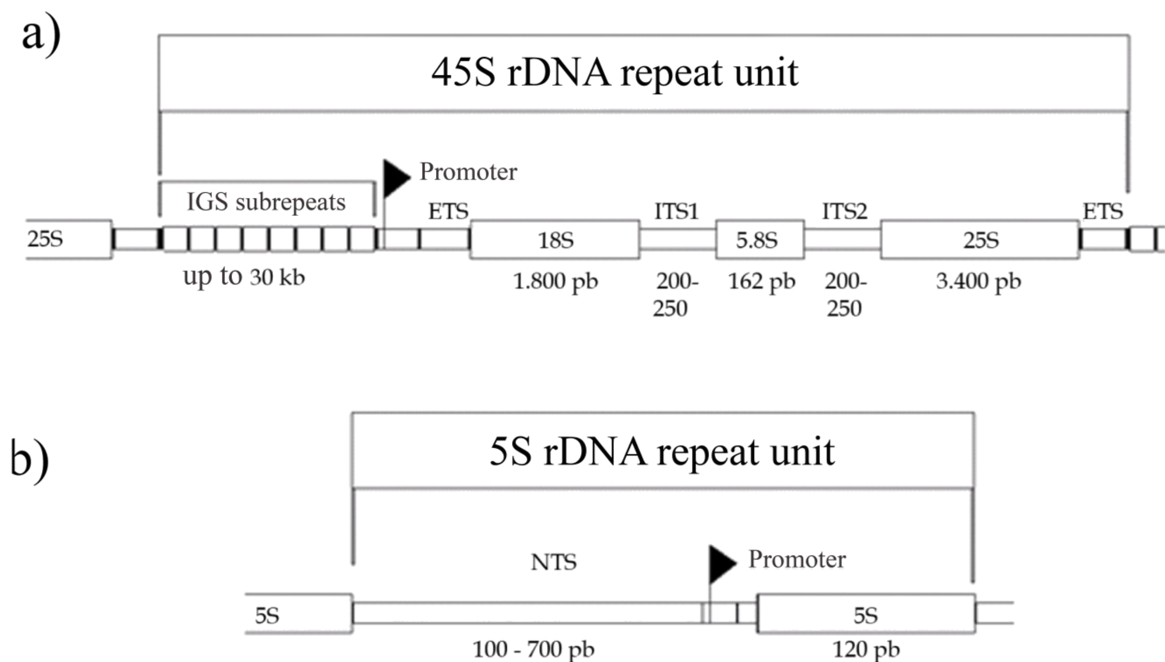


Fig. 4. Repeat units of 45S (**a**) and 5S (**b**) rDNA in eukaryotes and approximate length in base pairs. Based on Sastri et al. (1992), Ritland et al. (1993), Douet and Tourmente (2007), and Eickbush and Eickbush (2007).

Telomere sequences are localized at the chromosome ends. The *Arabidopsis*-like telomere repeat consist of arrays of 7-bp DNA (TTTAGGG) and has been investigated in many species, ranging from the green alga *Chlorella vulgaris* over mosses, ferns, and *Pinus* to many monocots and dicots (Lamb et al., 2012). So far, only a few Asparagales are known to lack the *Arabidopsis*-type repeat at the chromosome ends, instead having vertebrate-type telomere repeats, TTAGGG (Weiss-Schneeweiss et al., 2004; Lamb et al., 2012). For the

monocot genus *Allium* and the eudicot genus *Cestrum* (Solanaceae), the composition of the telomere sequences remains unknown (Lamb et al., 2012).

My study systems and research questions: Araceae and *Coccinia*

In my doctoral research I use three of the above-described tools to study the evolution of chromosome numbers, namely molecular phylogenetics, model-based character reconstruction, and FISH-labeling. I applied these tools to study chromosome evolution in the monocot family Araceae and to investigate the increase in size of sex chromosomes in the Cucurbitaceae genus *Coccinia.* One species in this genus, *C. grandis*, has the largest Y chromosome known in the land plants. Although its huge Y chromosome was first documented in 1952 (Kumar and Vishveshwaraiah, 1952), the species and its relatives were not studied with molecular studies until the beginning of the 21st century (Chapter 5). I now explain my choice of these two study systems.

Araceae are a large family – at least 3790 species in 118 genera (Boyce and Croat, 2011) – and their chromosome numbers range from $2n = 8$ to 168 (Cusimano et al., 2012a: Table S1; Sousa et al., 2014). Including my own new counts, chromosome counts are now available for 862 (26%) of the species (Cusimano et al., 2012a: Table S1 lists their names and the original references). Prior to my work, chromosome evolution in this family had been studied only by compiling chromosome numbers and discussing them in the context of morphology-based classifications (Petersen, 1993; Bogner and Petersen, 2007). The frequency of chromosome numbers in different clades of the family, or the clades' composition and relationships, were thus not considered in a reproducible (quantitative) way.

Two basic chromosome numbers (cf. pp. 5 and 6) had been suggested for the Araceae. Larsen (1969) and Marchant (1973) argued for $x = 7$, with higher numbers derived through ancient polyploidization event (genome duplication) or ascending dysploid series (increase or decrease of chromosomes numbers by rearrangements or fission). By contrast, Petersen (1993) hypothesized a base number of $x = 14$ because $2n = 28$ is especially common in the family. While the former hypothesis was put forward without the benefit of a phylogenetic framework, Petersen (1993) and Bogner and Petersen (2007) took into account morphological phylogenies (Grayum, 1990; Mayo et al., 1997). Nevertheless and as discussed above (p. 9), the use of "the most common number" or "the smallest chromosome number found in the

family" does not necessarily reflect or reconstruct the evolution of past chromosome changes that underlie current karyotypes (see the example of *Arabidopsis thaliana* on pp. 8 and 9). Criteria for inferring ancestral (perhaps no longer present) chromosome numbers from empirical counts could come from phylogenies, the relative frequencies of different haploid numbers in various species groups, cytogenetic work on closely related species, or, best, a combination of all such information.

To infer chromosome evolution in the Araceae in a reproducible manner, I used the model-based method of ancestral trait reconstruction developed by Mayrose et al. (2010) on a phylogeny for the family (113 species from 113 genera) that I slightly enlarged and modified, using four chloroplast markers (Chapter 2). The results suggest an ancestral haploid number to the family of a (my symbol for inferred ancestral numbers) = 16 or 18, higher than the previously hypothesized base numbers of x = 7 (Larsen, 1969; Marchant, 1973) or x =14 (Peterssen, 1993). I also inferred a limited role of polyploidization, while descending dysploidy (loss) is the most common event explaining the chromosome number reduction across the family tree (Cusimano et al., 2012a; Chapter 2). Given the inferred high ancestral haploid numbers, chromosome fusions (neutrally termed 'losses' in the models of Mayrose et al., 2010) must have been common during evolution of Araceae, which is tested in the paper in Chapter 3.

Although many Araceae species are in cultivation, molecular cytogenetics studies in this family only began with my doctoral research, initially focusing on a relatively derived group, namely *Typhonium*. *Typhonium* is a Southeast Asian genus of 50-60 species that has also been the focus of phylogenetic studies, natural geographic range, and diversification rate (Cusimano et al., 2010, 2012b; Chapter 3 and 4 of this thesis). At the start of my work, chromosome counts were available for 10 *Typhonium* species and ranged from 2n = 10 to 65. The genus is embedded within clades with chromosome numbers based on n = 13 or 14 (*Arisaema, Pinellia, Sauromatum, Biarum, Helicodiceros, Dracunculus,* and *Arum*), only *Theriophonum* has n = 8. In Cusimano et al. (2012a; Chapter 2), an ancestral chromosome number of a = 14 was inferred for the tribe Areae to which *Typhonium* belongs, and consequently, the low numbers found in this genus most likely represents a reduction.

After combining molecular cytogenetic and phylogenetic modeling in *Typhonium* to elucidate the evolution of its wide range of chromosome numbers, I tested the inferred past evolutionary changes by using FISH to search for the presence of interstitial telomere repeats

that might indicate chromosomal fusion, following my idea to test inferred dysploidy with FISH (p. 15 above). I greatly enlarged the phylogeny for the genus and its relatives to cover 96 taxa, and combined new and published chromosome counts to model evolutionary changes in chromosome complements at a finer scale. Ten species cultivated in the Botanical Garden of Munich were selected to perform FISH experiments, with the species chosen to represent the full range from $2n = 8$, the lowest number in family (newly reported in Chapter 3), to $2n = 24$.

Following my work on the 113-genus tree and the *Typhonium* group (Chapters 2 and 3), I decided to carry out cytogenetic analyses of telomere organization, focusing on early-diverging genera in the Araceae and on other genera of pivotal phylogenetic position never before studied (Chapter 4). The idea still was to search for signals of chromosome loss to test for cytogenetic traces of the dysploidy inferred in my modeling studies. So far, *Pinus* is the genus with the most conspicuous interstitial telomere FISH signals, with often up to four signals near the centromere and in interstitial positions (Fuchs et al., 1995; Lubaretz et al. 1996; Schmidt et al. 2000; Hizume et al. 2002; Islam-Faridi et al. 2007). I built another larger phylogeny from sequences of the plastid *trnL* intron and spacer, the *matK* gene and partial *trnK* intron, and the *rbcL* gene, this time for 173 species from 118 genera, and carried out an analysis of chromosome number evolution. I also performed FISH with three probes (5S rDNA, 45S rDNA, and *Arabidopsis*-like telomeres) on 14 species with $2n = 14$ to $2n = 60$.

Besides carrying out broad-scale analysis in the Araceae, I decided to work on the evolution of heteromorphic sex chromosomes, focusing on one species for which cultivated material and a phylogeny were available in our lab from the doctoral dissertation of Norbert Holstein (Holstein and Renner, 2010; Holstein and Renner, 2011a; Holstein and Renner, 2011b; Chapter 5). The species in question is *Coccinia grandis* from a genus with just 28 species that is phylogenetically close to cucumber and melon, both in the genus *Cucumis* (Schaefer and Renner, 2011; Fig. 5). I first reviewed published molecular cytogenetic studies on plant species with sex chromosomes and then used FISH, GISH, and C-banding on species of *Coccinia* cultivated in the green houses in Munich. In the flowering plants, heteromorphic (morphologically different) sex chromosomes are known from only 19 species belonging to four families; homomorphic sex chromosomes have been reported in 20 species belonging to 13 families (Ming et al., 2011). In angiosperms with heteromorphic sex chromosomes, the Y-chromosome is often larger than the X and the autosomes, and this has been attributed to the

accumulation of repetitive DNA (Shibata et al., 1999; Sakamoto et al., 2000; Cemark et al., 2008; Kejnovsky et al., 2009). In the land plants, *Marchantia polymorpha* (liverworts) and in *Cycas revoluta* (Cycadaceae) have the Y chromosome smaller than the X chromosome (Segawa et al., 1971; Ono, 1976; Okada et al., 2001).

Only little is known about the steps involved in the formation of sex chromosomes in plants compared with animals. Most molecular studies focus on the DNA composition and characterization of repetitive elements on X and Y chromosomes or on sex chromosomes versus the rest of the genome, development of specific sex chromosome probes and genetic mapping (*Carica* sp.: Liu et al., 2004; *Humulus* sp.: Divashuk et al., 2011; Grabowska-Joachimiak et al., 2011; *Rumex* sp.: Shibata et al., 1999; Steflova et al., 2013; *Silene* sp.: Lengerova et al., 2004; Filatov, 2005; Makova et al., 2007; Cermak et al., 2008; Macas et al., 2012). So far, only the *Silene latifolia* sex specific regions (MSY) and sex-linked genes are reasonably well studied, although the *Silene* genome (5.85 pg/2C in males) is huge and has therefore not been sequenced. By contrast, the *Coccinia grandis* genome is tiny, with 2C = 0.943 pg/2C (Sousa et al., 2013).

Based on a few molecular clock studies, it is thought that sex chromosomes in plants are young (Sousa et al., 2013: Table 3). The X and Y chromosomes of *Silene latifolia* may have diverged from each other between 8 and 24 Ma ago, in *Rumex* between 15-16 Ma ago, and in papaya between 0.5-2.2 Ma ago (Sousa et al., 2013: Table 3). Prior to my doctoral research, *Coccinia grandis* (incl. *C. indica*) had not been analyzed with molecular-cytogenetic methods, although experimental work had clearly established the sex-determining role of the single large Y chromosome found in males (Roy and Roy, 1971; for classic cytogenetic studies on this species see Chapter 5).

Chromosome counts are now available for six species of *Coccinia*, and so far only *C. grandis* has heteromorphic sex chromosomes. *Coccinia grandis* is about 3 Ma old (Fig. 5). My results show that the Y chromosome is twice as long as the largest autosome, resulting in a male/female C-value difference of 0.09 pg or 10% of the total genome (Chapter 5), compared to 8-9% in *Silene*. Its relatively small genome size, large Y chromosome, and phylogenetic proximity to the fully sequenced *Cucumis sativus* make *C. grandis* a promising model to study sex chromosome evolution.

Fig. 5. Modified from Yang et al. (2014). An asterisk marks species with whole genome data in GenBank. Photo of *C.grandis* from N. Holstein (personal communication).

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Chapter 2

Maximum likelihood inference implies a high, not a low, ancestral haploid chromosome number in the Araceae, with a critique of the bias introduced by “*x*”.

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Maximum likelihood inference implies a high, not a low, ancestral haploid chromosome number in Araceae, with a critique of the bias introduced by '*x*'

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• *Background and Aims* For 84 years, botanists have relied on calculating the highest common factor for series of haploid chromosome numbers to arrive at a so-called basic number, *x*. This was done without consistent (reproducible) reference to species relationships and frequencies of different numbers in a clade. Likelihood models that treat polyploidy, chromosome fusion and fission as events with particular probabilities now allow reconstruction of ancestral chromosome numbers in an explicit framework. We have used a modelling approach to reconstruct chromosome number change in the large monocot family Araceae and to test earlier hypotheses about basic numbers in the family.
• *Methods* Using a maximum likelihood approach and chromosome counts for 26 % of the 3300 species of Araceae and representative numbers for each of the other 13 families of Alismatales, polyploidization events and single chromosome changes were inferred on a genus-level phylogenetic tree for 113 of the 117 genera of Araceae.
• *Key Results* The previously inferred basic numbers $x = 14$ and $x = 7$ are rejected. Instead, maximum likelihood optimization revealed an ancestral haploid chromosome number of $n = 16$, Bayesian inference of $n = 18$. Chromosome fusion (loss) is the predominant inferred event, whereas polyploidization events occurred less frequently and mainly towards the tips of the tree.
• *Conclusions* The bias towards low basic numbers (*x*) introduced by the algebraic approach to inferring chromosome number changes, prevalent among botanists, may have contributed to an unrealistic picture of ancestral chromosome numbers in many plant clades. The availability of robust quantitative methods for reconstructing ancestral chromosome numbers on molecular phylogenetic trees (with or without branch length information), with confidence statistics, makes the calculation of *x* an obsolete approach, at least when applied to large clades.

Key words: Araceae, Bayesian inference, chromosome evolution, haploid chromosome number, dysploidy, maximum likelihood inference, polyploidy.

INTRODUCTION

Chromosome numbers in angiosperms vary from $n = 2$ (Tsvelev and Zhukova, 1974; Singh and Harvey, 1975; Sokolovskaya and Probatova, 1977; Erben, 1996) over $n = 250$ (Oginuma *et al.*, 2006) and $n = 298$ (Johnson *et al.*, 1989) to $n = 320$ (Uhl, 1978). The range in animals is similar (Crosland and Crozier, 1986; Imai *et al.*, 2002). Such drastic differences in chromosome number, sometimes even within small groups, raise questions about the evolutionary direction and frequency of the implied drastic genome rearrangements. Cytogenetic studies have shown that chromosome numbers can change due to fission, fusion or genome doubling (Guerra, 2008), and there is ample evidence that such changes can contribute to speciation. It has also been inferred that a large fraction of all plant species may have polyploid genomes (Stebbins, 1971; Goldblatt, 1980; Otto and Whitton, 2000; Ramsey and Schemske, 2002; Cui *et al.*, 2006; Soltis *et al.*, 2009; Wood *et al.*, 2009; Jiao *et al.*, 2011). Chromosome counts, however, exist only for 60 000 of the 300 000–352 000 species of flowering plants (Bennett, 1998; http://www.theplantlist.org/browse/A/). Most published numbers are listed in an electronic database for chromosome numbers, the 'Index of Plant Chromosome numbers' (http://mobot.mobot.org/W3T/Search/ipcn.html).

Given the incomplete knowledge of angiosperm chromosome numbers, evolutionary changes in chromosome numbers in most clades can only be estimated. Botanists do this by calculating a so-called basic, or monoploid, chromosome number, denoted *x*, to differentiate it from the haploid (usually the gametophytic) number *n* and the diploid (sporophytic or somatic) number 2*n*. The concept of *x* goes back to Langlet (1927) who explained it using *Aconitum* as an example; if different *Aconitum* species have $n = 8$, $n = 12$, $n = 16$ and $n =$ approx. 32, their inferred monoploid number *x* is 4 (Langlet, 1927: 7). Langlet's idea took off, at least in botany, where thousands of basic chromosome numbers have been inferred, even for poorly counted groups. Thus, for flowering plants, Raven (1975, p. 760) suggested $x = 7$ as 'characteristic of all major groups of both dicots and monocots except Caryophyllidae.' Similarly, base chromosome numbers of $x = 12$ or $x = 5$ and 6 have been suggested for Poaceae (reviewed in Hilu, 2004) and $x = 7$ or $x = 12$ for Triticeae (Heslop-Harrison, 1992; Luo *et al.*, 2009). Many further examples of divergent base numbers having been calculated for a clade could be cited (Soltis *et al.*, 2005; Blöch

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et al., 2009). Part of the reason why different researchers arrived at different base numbers (x) has to do with the unclear definition of x, with some treating it in Langlet's original sense as an algebraically discoverable highest common factor, others as 'the lowest *detectable* haploid number within a group of related taxa' (Stuessy, 2009: 264; italics ours), and yet others as 'the haploid number present in the initial population of a monophyletic clade' (Guerra, 2008: 340), i.e. an inferred number, since the 'initial population' will not usually have its chromosomes counted. How to make the inference is up to the investigator. Zoologists, in contrast, never became enamoured of the concept of an inferred base number x.

Criteria for inferring ancestral (perhaps no longer present) chromosome numbers from empirical counts could come from phylogenetic analyses, the relative frequencies of different haploid numbers in various species groups, cytogenetic work on closely related species or, best, a combination of all such information. Data from genomics and molecular–cytogenetic methods, such as fluorescence *in situ* hybridization (FISH)-marking chromosomes, are probably the best way to search for evidence of past chromosome number changes because they can identify synteny, fusion sites or unusual locations of centromeres, in turn providing evidence for duplications, fusions or losses (Bowers *et al.*, 2003; Lysak *et al.*, 2006; Peruzzi *et al.*, 2009). Such methods, however, may not be feasible in large clades or those with few species in cultivation.

In 2010, an approach was developed that moves the inference of chromosome number evolution to maximum likelihood (ML) character state reconstruction (Mayrose *et al.*, 2010). Mayrose *et al.* (2010) formulated probabilistic models describing the evolution of chromosome number across a phylogenetic tree. Their approach makes use of branch lengths as a proxy for time and of the frequencies of different numbers at the tips and in outgroups to infer transition rates between the different states. Ancestral chromosome numbers have previously sometimes been reconstructed using maximum parsimony (e.g. Soltis *et al.*, 2005: 178, 298–302). Parsimony, however, assigns all state transitions the same weight and disregards information contained in phylogenetic branch length, which tends to result in an underestimate of the number of transition events.

In this study we use the approach of Mayrose *et al.* (2010) to reconstruct ancestral haploid chromosome numbers in Araceae, a large and old family of monocotyledons. For a mainly tropical family, Araceae have a high number of chromosomes counts, with 862 (26 %) of their approx. 3300 species counted, including at least one species for most of the 117 genera (Petersen, 1989; Bogner and Petersen, 2007; Appendix; Supplementary Data Table S1 lists all species with their n and/or $2n$ counts and the respective references). Two basic chromosome numbers have been suggested for Araceae. Larsen (1969) and Marchant (1973) argued for $x = 7$, with higher numbers derived through ancient polyploidization events or ascending dysploid series. In contrast, Petersen (1993) hypothesized a base number of $x = 14$ because $2n = 28$ is especially common in the family. The former hypothesis was put forward without the benefit of a phylogenetic framework, but Petersen (and also Bogner and Petersen, 2007) took into account morphological phylogenetic analyses (Grayum, 1990; Mayo *et al.*, 1997).

Molecular phylogenetic work over the past few years has resulted in aroid relationships at the generic level becoming relatively clear (French *et al.*, 1995; Cabrera *et al.*, 2008; Cusimano *et al.*, 2011). We here use the most recent phylogenetic analysis of Araceae to infer chromosome evolution in the family, using the model-based approach of Mayrose *et al.* (2010), in both its ML and Bayesian implementations, the latter having the advantage that uncertainty in ancestral state probabilities is readily quantified. To test the power of their method, Mayrose *et al.* (2010) first used simulated data and then four exemplar plant clades (*Aristolochia*, *Carex*, *Passiflora* and *Helianthus*) with relatively densely sampled phylogenetic trees and chromosome counts. Sampling in these clades ranged from 11 to 100 % of the species in the genera. The Araceae data set analysed here represents an entire family that is larger and older by at least an order of magnitude. This poses challenges that we tried to address by experimentally modifying character codings to take into account uncertainties in the larger genera and the 13 outgroup families.

METHODS

Family and order phylogeny

The phylogenetic tree for Araceae on which ancestral chromosome numbers were inferred in this study is based on the six-plastid marker matrix of Cusimano *et al.* (2011). Clades are named as proposed in that study. We used the ML tree from that study or an ultrametric Bayesian tree newly obtained using BEAST v. 1·6·1 (Drummond and Rambaut, 2007). In BEAST, we used the GTR + G model with four rate categories, a mean substitution rate estimated from the data, and a pure-birth Yule model as the tree prior. The GTR + G model fit the data best, as assessed with Modeltest (Posada and Crandall, 1998). The analysis was run for 37 million generations, sampling every 1000th step. The burn-in fraction, i.e. the number of trees to be discarded before runs reached stationarity, was assessed using the Tracer v. 1·4·1 program (part of the BEAST package) and AWTY (Nylander *et al.*, 2008). For one set of analyses (below), we included only Araceae. For another, we included one exemplar each of the other families of Alismatales (Stevens, 2001 onwards), using branch lengths of 0·01 except for Tofieldiaceae (*Tofieldia*), which was the outgroup used by Cusimano *et al.* (2011) and had an empirical branch length.

Chromosome number coding

Total numbers of genera and species of Araceae were taken from the website Creating a Taxonomic eScience (CATE; http://www.cate-araceae.org/) and then updated by the Araceae specialist Josef Bogner (see Acknowledgements). Of the 117 currently recognized genera of Araceae, 29 are monospecific (and hence can be coded unambiguously for chromosome number), 19 have just two species, 31 have 3–10 species, 25 have 11–50 species and 13 have >50 species. Araceae chromosome counts were compiled from original literature

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(Supplementary Data Table S1, available online), checking the generic assignment of each species against the current classification and for synonymy. Chromosome numbers for four monotypic genera were contributed by J. Bogner and E. Vosyka (see Acknowledgements) and are newly reported here: *Filarum manserichense* Nicolson (M. Sizemore *s.n*., voucher in the herbarium M), *Hestia longifolia* (Ridl.) S. Y. Wong & P. C. Boyce (J. Bogner 3003, M), *Philonotion americanum* (A. M. E. Jonker & Jonker) S. Y. Wong & P. C. Boyce (J. Bogner 2911, M) and *Pichinia disticha* S. Y. Wong & P. C. Boyce (P. C. Boyce *s.n*., M; Supplementary Data Table S1). One genus was coded as unknown (X), namely the monotypic *Schottariella*, the chromosomes of which have not been counted. The presence of B chromosomes was not coded. Overall, our phylogenetic analysis includes 113 of the 117 accepted genera of Araceae, with 112 of them coded for haploid chromosome number (Appendix).

Chromosome numbers were coded in three ways to address the problem of genera with more than one chromosome number. First, we coded all reported numbers for each genus, regardless of frequency in different species, but excluding odd numbers (Appendix, column 5; Supplementary Data Table S1). This resulted in 55 genera coded as polymorphic. Our second coding scheme ('reduced polymorphism' coding) took into account the frequency of different numbers and treated the most common as the ancestral state (Appendix, column 7; Supplementary Data Table S1). For example, Lemnoideae have many different chromosome numbers, but $n = 20$ is especially common (Landolt, 1986; Appendix, Supplementary Data Table S1). For genera with numbers suggesting different ploidy levels, we used the lowest haploid chromosome number (e.g. *Arum*). Polymorphisms could thus be reduced to two states (chromosome numbers) per genus or even a single haploid number, leaving 34 instead of 55 genera with polymorphic numbers. In a third coding scheme ('informed' coding), we took into account molecular phylogenetic analyses for the genera *Philodendron* (Gauthier *et al.*, 2008), *Biarum* and *Typhonium* (Cusimano *et al.*, 2010), and assigned the state (chromosome number) found in the early-branching species to the entire genus. The numbers thus inferred were compared with those inferred by Bogner and Petersen (2007). This third approach left just ten genera coded as polymorphic with maximally two states (Appendix, column 8; Supplementary Data Table S1, Supplementary Data Figs S1 and S2). In this third scheme, *Lazarum*, a genus of 23 species with a few chromosome counts and insufficient phylogenetic information (Matthew Barrett, Botanic Gardens & Parks Authority, West Perth; personal communication, 2011) was coded as 'unknown' (X) because no ancestral haploid number could be inferred. In all cases, changes among character states (i.e. chromosome numbers) were assigned equal probability.

The remaining families of Alismatales were coded as follows: Alismataceae $n = 7$, 8; Aponogetonaceae $n = 12$, 16, 19; Butomaceae $n = 7$, 8, 10, 11, 12; Cymodoceaceae $n = 7$, 8, 10, 14, 15; Hydrocharitaceae $n =$ notably variable; Juncaginaceae $n = 6$, 8, 15; Maundiaceae only *Maundia triglochinoides*, no chromosome count reported; Posidoniaceae $n = 10$; Ruppiaceae $n = 8$–12, 15; Potamogetonaceae $n = 7$, 12, 14–18; Scheuchzeriaceae $n = 11$; Tofieldiaceae $n = 15$; Zosteraceae $n = 6$, 9, 10 (numbers from Stevens, 2001 onwards). Those of these families with more than one number listed by Stevens were coded as polymorphic in all analyses. The above-described three coding schemes were first run on the phylogenetic tree that included only Araceae and then on the tree that included the 13 outgroups, resulting in six analyses (labelled A1–A6 in Table 1).

Inference of chromosome number change

For ML and Bayesian phylogenetic inferences of ancestral haploid chromosome numbers, we relied on the chromEvol program v. 1·2 of Mayrose *et al.* (2010; http://www.zoology.ubc.ca/prog/ chromEvol.html). This implements eight models of chromosome number change (Table 2), two more than described in the original paper. The models include the following six parameters: polyploidization (chromosome number duplication with rate ρ, 'demi-duplication' or triploidization with rate μ) and dysploidization (ascending, chromosome gain rate λ; descending, chromosome loss rate δ) and two linear rate parameters, λ_1 and δ_1, for the dysploidization rates λ and δ, allowing them to depend on the current number of chromosomes. Four of the models have a constant rate, whereas the other four include the two linear rate parameters. Both model sets also have a null model that assumes no polyploidization events. We fitted all models to the data, each with 10 000 simulations to compute the expected number of changes of the four transition types along each branch. The maximum number of chromosomes was set to 10× higher then the highest number found in the empirical data, and the minimum number was set to 1. The null hypothesis (no polyploidy) was tested with likelihood ratio tests using the Akaike information criterion (AIC).

We also ran an analysis, using the informed polymorphism-coding scheme, but excluding *Calla* because of its unclear relationships in Araceae (Cusimano *et al.*, 2011). For a final sensitivity test, we again used the informed coding scheme but the non-ultrametric ML phylogenetic tree from Cusimano *et al.* (2011) instead of the ultrametric tree used in the remaining analyses.

RESULTS

The results obtained in the six analyses (A1–A6) are summarized in Table 1. The three-parameter constant-rate model (Mc2), with the chromosome duplication rate equal to the demi-duplication rate, was the best explanation of the empirical data in all analyses. All analyses rejected the null model of no polyploidy with high significance ($P < 0{\cdot}999$). The inferred rates of change, chromosome numbers at nodes (and their probability) and numbers of events were similar regardless of which of the three schemes for polymorphism coding was applied. We therefore show the results obtained from Bayesian and ML analyses with the most conservative coding scheme, namely the one including all polymorphisms and all outgroups (Table 1, A1; Figs 1 and 2). For comparison, the results from analysis A6, without outgroups and the phylogenetically informed coding (Appendix, column 8), can be found in Supplementary Data Figs S1 and S2.

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TABLE 1. *Results from the six analyses (A1–A6) carried out to infer chromosome number changes in Araceae under Bayesian and maximum likelihood optimization*

		Coding scheme						Rate parameters				Events inferred with PP >0·5				Chromosome no. at Araceae root node			Chromosome no. ranges at Araceae root node			
Analysis	Tree: outgroups	All poly.	Red. poly.	Inf.	Best model	LogLik	AIC	δ	λ	ρ	μ	Losses	Gains	Dupl.	Demi.	Bayes: Best *n*; *PP*	Bayes: 2nd best *n*; *PP*	ML	*n*	Sum *PP*	*n*	Sum *PP*
A1	+	+			Mc2	−219·5	445	45·9	3·9	6·9	–	98·1	8·4	14·3	14·3	18; 0·18	16; 0·16	16	16–18	0·5	8–18	0·9
A2	+		+		Mc2	−236·4	478·9	56·4	0	6·3	–	112·2	0	11·5	13	18; 0·26	17; 0·13	16	17–19	0·51	10–20	0·85
A3	+			+	Mc2	−245·7	497·3	58·2	0	5·7	–	120·1	0	11·9	13·9	18; 0·26	19; 0·12	17	17–19	0·52	10–20	0·9
A4	–	+			Mc2	−196·6	399·1	50·4	1·8	6·6	–	86·6	3·2	10·5	9·3	18; 0·38	17; 0·3	17	17–19	0·86		
A5	–		+		Mc2	−213·2	432·4	53·6	0	5·6	–	87·2	0	9·8	9·4	18; 0·42	17; 0·23	17	17–19	0·9		
A6	–			+	Mc2	−222·4	450·7	58·1	0	5·4	–	94·4	0	9·7	10·5	18; 0·37	19; 0·34	18	17–19	0·85		

Only the best-fitting models are shown. Tree (column 2) refers to whether outgroups were included or not; coding scheme refers to how genera with polymorphic haploid chromosome numbers were coded. All poly., all chromosome number polymorphism coded (scheme 1); Red. poly., reduced polymorphism coding (scheme 2); Inf., phylogenetically informed coding (scheme 3). Best model, Mc2 (constant rate model with duplication rate ρ and demi-duplication rate μ; compare Table 2); Logarithmic likelihood (LogLik) and AIC scores; rate parameters (δ = chromosome loss rate, λ = chromosome gain rate, ρ = duplication rate, μ = demi-duplication rate); frequency of the four possible event types with a posterior probability (PP) > 0·5; haploid chromosome number inferred at the root node under Bayesian optimization with the respective *PP*, and under maximum likelihood (ML). The last column shows the chromosome number range inferred for the root node, each with its *PP*.

The loss rate δ ranges from 45·9 (Table 1, A1) to 58·2 (A3), and the polyploidization rate $\rho = \mu$ from 5·4 (A6) to 6·9 (A1). A gain rate λ is inferred only for models A1 (3·9) and A4 (1·8, analyses with all polymorphisms coded). The number of events inferred with a probability of >0·5 is higher in the analyses using the tree with outgroups than in that without outgroups, simply because it has more branches. Inferred chromosome loss events range from 98·1 (A1) to 120·1 (A3), duplications from 11·5 (A2) to 14·3 (A1) and demi-duplications from 13 (A2) to 14·3 (A3)); in A1, 8·4 chromosome gain events were inferred, whereas, in the tree without outgroups, the number of losses ranges from 86·6 (A4) to 94·4 (A6), that of duplications from 9·7 (A6) to 10·5 (A4) and that of demi-duplications from 9·3 (A4) to 10·5 (A6); finally in A4, 3·2 chromosome gain events were inferred (Table 1, Fig. 1 and Supplementary Data Fig. S1, Bayesian inference). In the Bayesian analyses, the haploid chromosome number at the root with the highest posterior probability (*PP*) was $n = 18$, and support for this number was higher in analyses without outgroups (0·37–0·42) than in those with outgroups (0·18–0·26, Table 1). Similarly, a range of $n = 17–19$ at the root node had a *PP* of ≥0·85 without outgroups, but only ≤0·52 when outgroups were included (Table 1). A broader range of ancestral numbers [$n = 8–18$ (A1); $n = 10–20$ (A2, A3)] could be inferred with higher *PP* (>0·85, Table 1, Fig. 1 and Supplementary Data Fig. S1). In the ML analyses with outgroups (Fig. 2), the most likely haploid number at the root was $n = 16/17$, and without outgroups it was 17/18 (Table 1; Supplementary Data Fig. S2).

To describe inferred chromosome evolution in Araceae, we focus on the Bayesian inference of the most conservative analysis scheme A1 depicted in Fig. 1. Starting from the root node, chromosome numbers decreased, becoming $n = 15$ along the branch leading to the *Spirodela* clade ($PP = 0·32$; $n = 16$: $PP = 0·29$), $n = 15$ in Araceae ($PP = 0·55$; $n = 14$: $PP = 0·21$), and $n = 14$ in the *Podolasia* clade ($PP = 0.\ 62$; $n = 15$: $PP = 0·24$). The number $n = 14$ is inferred with increasing probability as one moves up the phylogenetic tree towards the present. It has 0·77 *PP* in Aroideae and 0·99 *PP* in the *Ambrosina* clade.

Increases in chromosome number are inferred as deriving from (demi-) duplication events, never via single chromosome gains (centric fission), whereas decreases in chromosome number are inferred as resulting from chromosome loss (fusion). The most likely events (*PP* >0·5) predicted by the best-fitting model are descending dysploidy (98·1 events), and these are inferred both on branches leading to major clades (e.g. Pothoideae, Lasioideae and Spathicarpeae) and on terminal branches. The only chromosome gain event in Araceae inferred with high probability occurred on the branch leading to *Scaphispatha* ($n = 14$). Polyploidization events (29 in total, Fig. 1) occur mainly towards the tips of the tree (*Gymnostachys*, *Alloschemone*, *Urospatha*, *Anubias*, *Montrichardia*, Cryptocoryneae, *Calla*, *Filarum* and *Peltandra*). Only three polyploidization events are inferred deeper in the tree: a genome duplication on the branch leading to the *Rhaphidophora* (Fig. 1) clade (from $n = 15$ to $n = 30$), a demi-duplication on the branch leading to the *Zantedeschia* clade (from $n = 14$ to $n = 21$) and one

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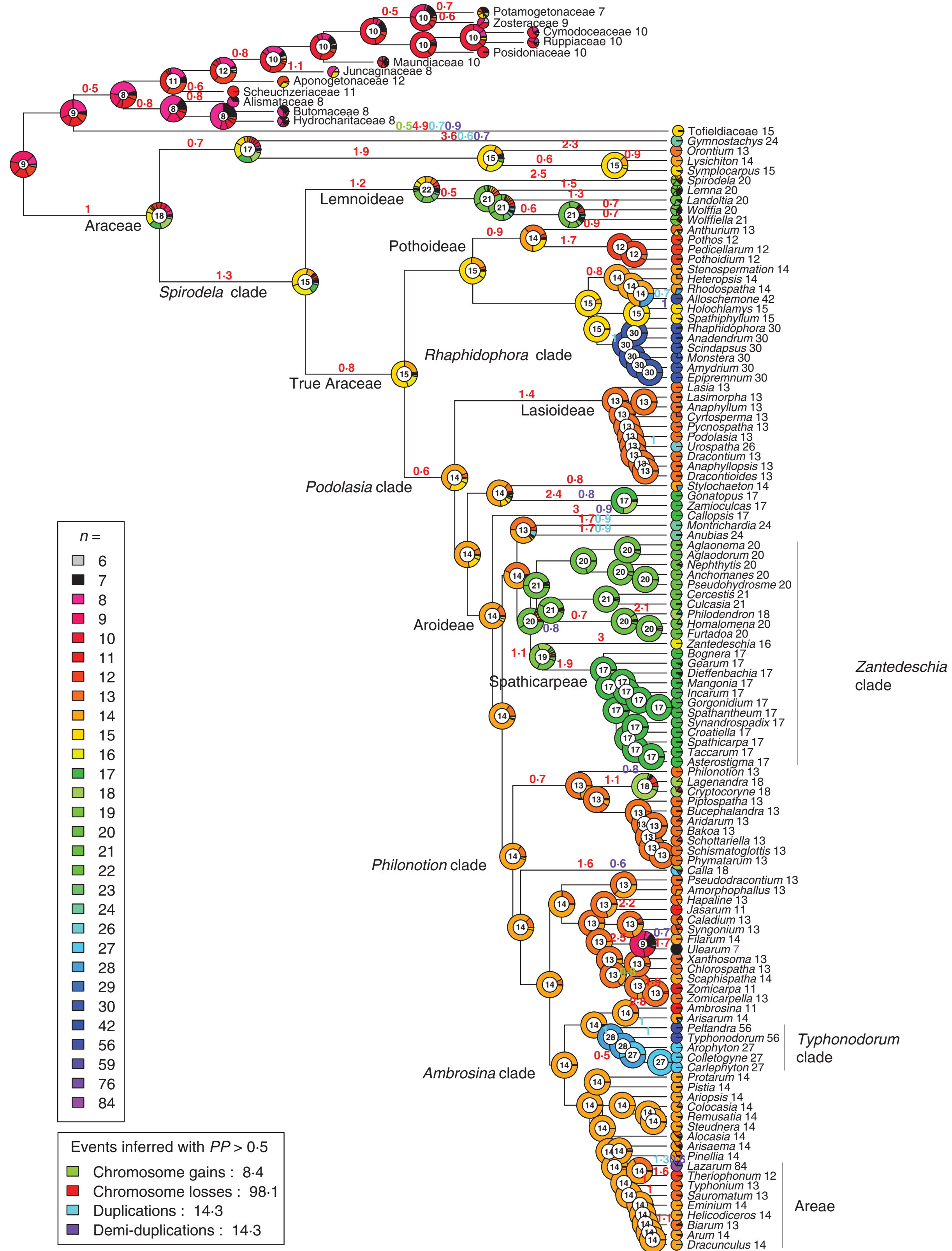

FIG. 1. Chromosome number evolution in Araceae inferred under Bayesian optimization, with outgroups included and all polymorphic chromosome states coded (analysis A1 in Table 1). Pie charts at nodes and tips represent the probabilities of the inferred chromosome number(s); numbers inside charts have the highest probability. Numbers at the tips are chromosome numbers inferred with the highest probability, i.e. the inferred ancestral haploid chromosome number for each genus. Numbers above branches represent the inferred frequency of those of the four possible events (gains, losses, duplications and demi-duplications) that had a probability >0·5. The colour coding of chromosome numbers and event types is explained in the insets.

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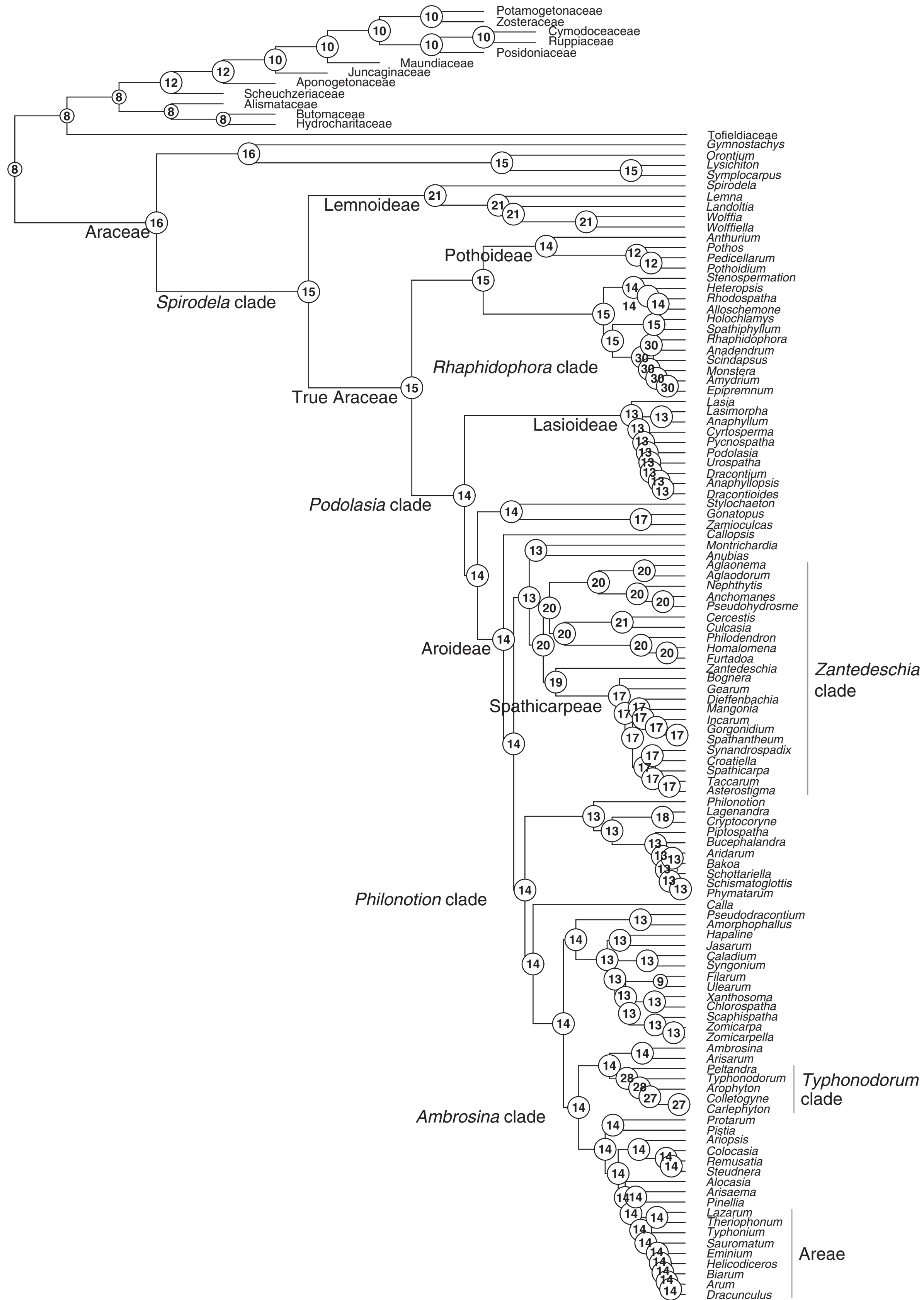

FIG. 2. Chromosome number evolution in Araceae inferred under maximum likelihood optimization, with outgroups included and all polymorphic chromosome states coded (analysis A1 in Table 1).

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TABLE 2. *The eight models of chromosome number evolution implemented in the software of Mayrose et al. (2010), indicating the considered parameter estimates (δ, λ, ρ, μ, δ_1, λ_1), the number of parameters included, and, in the case of μ, with which condition*

Model	δ	λ	ρ	μ	δ_1	λ_1	No. of parameters
Mc1	+	+	+	–	–	–	3
Mc2	+	+	+	$\rho = \mu$	–	–	3
Mc3	+	+	+	$\rho \neq \mu$	–	–	4
Mc0	+	+	$\rho = 0$	$\mu = 0$	–	–	2
Ml1	+	+	+	–	+	+	5
Ml2	+	+	+	$\rho = \mu$	+	+	5
Ml3	+	+	+	$\rho \neq \mu$	+	+	6
Ml0	+	+	$\rho = 0$	$\mu = 0$	+	+	4

Mc indicates models with constant rates, and Ml models that include linear rate parameters (δ_1, λ_1). Zero indicates the respective null model.

duplication event on the branch leading to the *Typhondorum* clade (from $n = 14$ to $n = 28$).

Results of the two additional analyses (inclusion/exclusion of *Calla*; ultrametric or non-ultrametric trees) did not yield results substantially different from those obtained in analysis A6 and shown in Supplementary Data Fig. S1. Model Mc2 remained the best-fitting model, and chromosome number reconstructions at nodes and change rates were similar.

DISCUSSION

The results presented here provide an example of the power of ML-based or Bayesian inference of chromosome number changes. The new approach, which distinguishes (and separately infers) chromosome gains, losses, polyploidization and demi-ploidization, not only reconstructs numbers at particular phylogenetic nodes, but also infers rates of change throughout the phylogenetic tree. Equally importantly, Bayesian *PP*s yield a statistically well-understood measure of confidence in the results. Most previous ancestral chromosome numbers, in contrast, have been inferred without confidence assessment (examples and critical discussion in Soltis *et al.*, 2005). The experiments we carried out with the different coding schemes for genera polymorphic for chromosome number revealed surprising robustness of the states inferred at interior nodes, although as expected the inclusion or exclusion of outgroups (in our case 13 families) affected the number inferred for the basal-most node, albeit only slightly (Table 1). The results of the present study further confirm that model-based chromosome inference works well even with large data matrices; the largest of the four matrices analysed by Mayrose *et al.* (2010) had 107 terminals, and the present tree had 126.

Chromosome fusion (loss) appears to be the predominant pattern in the evolution of chromosome number in Araceae; polyploidization events are much less frequent and apparently occurred mainly towards the tips of the tree. However, ancient polyploidization events may be harder to detect than recent ones, because of the genomic restructuring that follows polyploidization. Only detailed studies, perhaps involving chromosome painting techniques, will reveal how rapid intergenomic rearrangements have occurred after genome doubling, perhaps especially following hybridization (Hayasaki *et al.*, 2000; Lim *et al.*, 2008; Peruzzi *et al.*, 2009; Tu *et al.*, 2009).

In general, basic chromosome numbers inferred according to Langlet's (1927) approach, as the lowest detectable or somehow calculated haploid number within a group of related taxa, will be low, simply because of the way they are arrived at (see Introduction for Langlet's original example). For Araceae, the hypothesized ancestral numbers were $x = 14$ or $x = 7$ (Larsen, 1969; Marchant, 1973; Petersen, 1993). The present study instead inferred an ancestral haploid number of $n = 16$ (under ML) or $n = 18$ (with Bayesian inference) and, moreover, an evolutionary trend from higher to lower numbers, rather than the other way around. One needs to keep in mind that none of the earlier studies (Larsen, 1969; Marchant, 1973; Petersen, 1993) included Lemnoideae in Araceae, a taxonomic difference that greatly affects the range of chromosome numbers found in early-diverging clades (Figs 1 and 2). It is also likely that the high frequency of $2n = 28$ in the well-counted unisexual Aroideae unduly influenced the hypotheses about x being 7 or 14. Finally, the earlier hypotheses were developed without the relatively complete and solid phylogenetic information that is available today.

Nevertheless, any inferences about character evolution from a taxon sampling of just 112 representatives, however well coded their states may be, must be regarded with caution. Every genus with more than one species must have its own, perhaps complex, history of cytogenetic change. It is also conceivable that dysploidy rates might change in different parts of the tree (e.g. in clades of taxa living in different environments) and that relatively derived and rapidly radiating clades, perhaps with frequent hybridization, might have different rates of polyploidization than older, genetically isolated groups. The phylogenetically informed coding scheme (our scheme three) may be the best way of coding ancestral haploid chromosome numbers in larger clades (here genera), an idea that could be tested by cytological work in small genera with solid phylogenetic hypotheses, such as *Arum* (e.g. Espíndola *et al.*, 2010).

Given the inferred high ancestral haploid numbers, chromosome fusions (neutrally termed 'losses' in the models of Mayrose *et al.*, 2010) must have been common during evolution of Araceae. This hypothesis now needs to be tested. Large chromosomes in Araceae, with distally positioned centromeres, may be the result of fusion between smaller metacentric chromosomes (Petersen, 1993). If so, one expects to find interstitial telomeric sites. With probes, using primer pairs homologous to the basic plant telomeric repeats, one can visualize these regions (Ijdo *et al.*, 1991; Weiss-Schneeweiss *et al.*, 2004). Such chromosome preparations are now being carried out in our laboratory on *Typhonium* species with suspected chromosome fusion (predicted from high or low chromosome numbers in species of known phylogenetic relationships).

The results of the present study suggest that quantitative methods for inferring ancestral haploid numbers should replace inferences that rely on algebraically finding the greatest common factor for a series of numbers or on interpreting the lowest available haploid count as the ancestral condition.

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The new approaches also yield a measure of statistical confidence or estimates of the rates of polyploidization, fusion or fission, We suggest that the concept '*x*', which sets botanists apart from zoologists, be retained only in the context of small species groups in which the history of polyploidy is known in detail (Vanzela *et al.* 2003).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: chromosome counts for species of Araceae with references. Figure S1: chromosome number evolution in Araceae inferred under Bayesian optimization, with phylogenetically informed coding and outgroups excluded. Figure S2: chromosome number evolution in Araceae inferred under maximum likelihood optimization, with phylogenetically informed coding and outgroups excluded.

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APPENDIX

The 117 genera of Araceae with number of species, number and percentage of species with chromosome counts, diploid chromosome numbers and coded ancestral haploid chromosome numbers in the three coding schemes used in this study (see Methods). X = unknown.

	Genera	Spp. number	Spp. counted	%	Counted diploid chromosome numbers 2*n* =	All polymorphic *n* =	Reduced polymorphic *n* =	Informed *n* =
1	*Aglaodorum*	1	1	100	40	20	20	20
2	*Aglaonema*	23	6	26	14, 40, 100	7, 20, 50	7, 20, 50	20
3	*Alloschemone*	2	1	50	84	42	42	42
4	*Alocasia*	107	17	16	24, 26. 28, 40,42, 56, 68, 70, 84	12, 13, 14, 20, 21, 28, 34, 35, 42	12, 13, 14, 20, 21, 28, 34, 35, 42	14
5	*Ambrosina*	1	1	100	22	11	11	11
6	*Amorphophallus*	196	47	24	26, 28, 39	13, 14	13, 14	13
7	*Amydrium*	5	2	40	60	30	30	30
8	*Anadendrum*	11	3	27	60	30	30	30
9	*Anaphyllopsis*	3	1	33	26	13	13	13
10	*Anaphyllum*	2	2	100	26	13	13	13
11	*Anchomanes*	6	3	50	40	20	20	20
12	*Anthurium*	903	171	19	14, 20, 24, 26, 28, 29, 30 + Bs, 34, 36, 40, 48, 49, 56, 60, 84, approx. 90, approx. 124	7, 13, 15, 17, 18, 30	7, 13, 15, 17, 18, 30	15
13	*Anubias*	8	8	100	48	24	24	24
14	*Apoballis*	12	6	50	26, 39, 56	13, 28	13	13
15	*Aridarum*	10	4	40	24, 26	12, 13	12, 13	12, 13
16	*Ariopsis*	2	1	50	28, 84, 86	14, 42, 43	14	14
17	*Arisaema*	150	97	65	20, 24, 26, 28, 32,42, 48, 52, 56, 64, 70, 72, 84, 112, 140, 168	10, 12, 13, 14, 16, 21, 24, 26, 28, 32, 42, 56, 70, 84	10, 12, 13, 14, 16, 21, 24, 26, 28, 32, 42, 56, 70, 84	14
18	*Arisarum*	4	2	50	14, 28, 42, 52, 56	7, 14, 21, 26, 28	7, 14, 21, 26, 28	14
19	*Arophyton*	7	6	86	38, 40, 54, approx. 76	19, 20, 27	19, 20, 27	19
20	*Arum*	29	26	90	28, 29, 30, 42, 56, 63, 70, 84	14, 15, 21, 28, 35, 42	14	14
21	*Asterostigma*	8	2	25	34	17	17	17
22	*Bakoa*	2	2	100	26	13	13	13
23	*Biarum*	21	12	57	16, 18, 22, 24, 26, 32, 36, 40, 74, approx. 96, 98, 108	8, 9, 11, 12, 13, 16, 18, 20, 37, 49, 54	8, 9, 11, 12, 13, 16, 18, 20, 37, 49, 54	13
24	*Bognera*	1	1	100	34	17	17	17
25	*Bucephalandra*	3	3	100	26	13	13	13
26	*Caladium*	12	6	50	19, 22, 26, 28, 30	11, 13, 14, 15	11, 13, 14, 15	13, 14
27	*Calla*	1	1	100	36, 54, 60, 72	18, 27, 30, 36	18	18
28	*Callopsis*	1	1	100	36	17	17	17
29	*Carlephyton*	3	3	100	54, 108	27, 54	27	27
30	*Cercestis*	10	6	60	approx. 36, 42	21	21	21
31	*Chlorospatha*	28	2	7	26	13	13	13
32	*Colletogyne*	1	1	100	44, 46, 54	22, 23, 27	27	27
33	*Colocasia*	16	5	31	26, 28, 30, 36, 38, 42, 44, 46, 48, 52, 58, 84, 116	13, 14, 15, 18, 19, 21, 22, 23, 24, 26, 42, 58	13, 14, 15, 18, 19, 21, 22, 23, 24, 26, 42, 58	14
34	*Croatiella*	1	1	100	34	17	17	17
35	*Cryptocoryne*	60	64	107	20, 22, 28, 30, 33, 34, 36, 42, 54, 56, 66, 68, 70, 72, 85, 88, 90, 102, 112, approx. 132	10, 11, 14, 15, 17, 18, 21, 27, 28, 33, 34, 35, 36, 44, 45, 51, 56	10, 11, 14, 15, 17, 18, 21, 27, 28, 33, 34, 35, 36, 44, 45, 51, 56	17, 18
36	*Culcasia*	24	9	38	approx. 40, 42	21	21	21
37	*Cyrtosperma*	12	4	33	24, 26	12, 13	12, 13	13
38	*Dieffenbachia*	57	14	25	34, 36, 40, 44, 68	17, 18, 20, 22, 34	17	17
39	*Dracontioides*	2	1	50	26	13	13	13
40	*Dracontium*	24	5	21	26	13	13	13
41	*Dracunculus*	2	2	100	28, 32	14, 16	14	14
42	*Eminium*	9	3	33	28	14	14	14
43	*Epipremnum*	15	3	20	60, 70, 84	30, 35, 42	30, 35, 42	30
44	*Filarum*	1	1	100	28	14	14	14
45	*Furtadoa*	2	1	50	40	20	20	20
46	*Gearum*	1	1	100	34, 68	17, 34	17	17
47	*Gonatopus*	5	4	80	34, approx. 68	17	17	17
48	*Gorgonidium*	8	3	38	34	17	17	17
49	*Gymnostachys*	1	1	100	48	24	24	24
50	*Hapaline*	8	2	25	26, 28	13, 14	13, 14	13, 14

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TABLE *Continued*

	Genera	Spp. number	Spp. counted	%	Counted diploid chromosome numbers 2*n* =	All polymorphic *n* =	Reduced polymorphic *n* =	Informed *n* =
51	*Helicodiceros*	1	1	100	56	14	14	14
52	*Hestia*	1	1	100	26	13	13	13
53	*Heteropsis*	17	1	6	26–28	13, 14	13, 14	14
54	*Holochlamys*	1	1	100	30, 60	15	15	15
55	*Homalomena*	117	24	21	38, 40, 42, 56	19, 20, 21, 28	19, 20, 21, 28	20
56	*Incarum*	1	1	100	34	17	17	17
57	*Jasarum*	1	1	100	22	11	11	11
58	*Lagenandra*	15	14	93	32, 36, approx. 72	16, 18	16, 18	18
59	*Landoltia*	1	1	100	40,46, 50	20, 23, 25	20	20
60	*Lasia*	2	1	50	26	13	13	13
61	*Lasimorpha*	1	1	100	26	13	13	13
62	*Lazarum*	23	2	9	approx. 118, 130, 152, approx. 160,168	59, 65, 76, 84	59, 65, 76, 84	X
63	*Lemna*	13	11	85	20, 30, 36, 40, 42, 44, 50, 60, 63, 64, 70, 80, 84, 126	10, 15, 18, 20, 21, 22, 25, 30, 32, 35, 40, 42, 63	20	20
64	*Lysichiton*	2	2	100	28	14	14	14
65	*Mangonia*	2	1	50	34	17	17	17
66	*Monstera*	39	5	13	24, 56, 58, 60	12, 28, 29, 30	30	30
67	*Montrichardia*	2	1	50	48	24	24	24
68	*Nephthytis*	6	5	83	36, 40, 60	18, 20, 30	18, 20	18, 20
69	*Ooia*	2	1	50	26	13	13	13
70	*Orontium*	1	1	100	26	13	13	13
71	*Pedicellarum*	1	1	100	24	12	12	12
72	*Peltandra*	2	1	50	112	56	56	56
73	*Philodendron*	483	31	6	26, 30, 32, 34, 36, 48, 54	13, 15, 16, 17, 18, 24, 27	13, 15, 16, 17, 18, 24, 27	17, 18
74	*Philonotion*	3	1	33	26	13	13	13
75	*Phymatarum*	1	1	100	26, 28	13	13	13
76	*Pichinia*	1	1	100	26	13, 14	13, 14	13
77	*Pinellia*	9	9	100	20, 26, 28, 39, 42, 52, 54, 72, 78, 90, 91, 99, 104, 108, 115, 116, 117, 128, 129	10, 13, 14, 21, 26, 27, 36, 39, 45, 52, 54, 58, 64	10, 13, 14, 21, 26, 27, 36, 39, 45, 52, 54, 58, 64	13
78	*Piptospatha*	10	6	60	26, 39	13	13	13
79	*Pistia*	1	1	100	14, 28	7, 14	7, 14	14
80	*Podolasia*	1	1	100	26	13	13	13
81	*Pothoidium*	1	1	100	24	12	12	12
82	*Pothos*	57	3	5	24, 36, 60	12, 18, 30	12	12
83	*Protarum*	1	1	100	28	14	14	14
84	*Pseudodracontium*	7	2	29	26	13	13	13
85	*Pseudohydrosme*	2	1	50	approx. 40	20	20	20
86	*Pycnospatha*	2	2	100	26	13	13	13
87	*Remusatia*	4	4	100	20, 28, 30, 42, 56	10, 14, 15, 21, 28	10, 14, 15, 21, 28	14
88	*Rhaphidophora*	98	8	8	26, 42, 54, 56, 60, approx. 120	13, 21, 27, 28, 30	28, 30	28, 30
89	*Rhodospatha*	29	3	10	28, 56, 60	14, 28, 30	14, 28	14
90	*Sauromatum*	9	7	78	26, 52, 54, 104	13, 26, 27, 52	13	13
91	*Scaphispatha*	2	1	50	28	14	14	14
92	*Schismatoglottis*	100	15	15	26, 30, 39, 52	13, 15, 26	13	13
93	*Schottariella*	1	0	0	–	X	X	X
94	*Scindapsus*	35	8	23	48, 60 (42, 56, 58, 64, 70, 112), approx. 110	28, 30	28, 30	28, 30
95	*Spathantheum*	2	2	100	34	17	17	17
96	*Spathicarpa*	4	1	25	34	17	17	17
97	*Spathiphyllum*	49	9	18	30, 60	15, 30	15	15
98	*Spirodela*	3	2	67	20, 30, 32, 36, 38, 40, 50, 80	10, 15, 16, 18, 19, 20, 25, 40	15, 20	15, 20
99	*Stenospermation*	50	4	8	28	14	14	14
100	*Steudnera*	9	4	44	28, 36,56	14, 18, 28	14	14
101	*Stylochaeton*	18	4	22	28, 56	14, 28	14, 28	14
102	*Symplocarpus*	5	2	40	30, 60	15, 30	15, 30	15
103	*Synandrospadix*	1	1	100	34	17	17	17
104	*Syngonium*	35	9	26	22, 24, 26, 28	11, 12, 13, 14	14	14
105	*Taccarum*	6	1	17	34	17	17	17
106	*Theriophonum*	5	5	100	16, 24, 32 (14, 18)	8, 12, 16	8	8
107	*Typhonium*	68	8	12	10, 16, 18, 20, 26, 36, 52,65	5, 8, 9, 10, 13, 18, 26	5, 6, 7, 8, 9, 10, 13, 18, 26	8, 13

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Table *Continued*

	Genera	Spp. number	Spp. counted	%	Counted diploid chromosome numbers 2*n* =	All polymorphic *n* =	Reduced polymorphic *n* =	Informed *n* =
108	*Typhonodorum*	1	1	100	112	56	56	56
109	*Ulearum*	2	2	100	14	7	7	7
110	*Urospatha*	12	1	8	52	26	26	26
111	*Wolffia*	11	8	73	16, 20, 22, 23, 30, 40, 42, 46, 50, 60, 62, 63, 70, 80	8, 10, 11, 15, 20, 21, 23, 25, 30, 31, 35, 40	20	20
112	*Wolffiella*	10	7	70	20, 40, 42, 50, 70	10, 20, 21, 25, 35	20	20
113	*Xanthosoma*	75	11	15	22, 26, 39, 52	11, 13, 26	11, 13, 26	13
114	*Zamioculcas*	1	1	100	34	17	17	17
115	*Zantedeschia*	8	7	88	32	16	16	16
116	*Zomicarpa*	3	2	67	20, 22	10, 11	10, 11	10
117	*Zomicarpella*	2	1	50	26	13	13	13
	Total	3309	847					
	Mean			61				

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SUPPLEMENTARY DATA

FIG. S1. Chromosome number evolution in Araceae inferred under Bayesian optimization, with phylogenetically informed coding and outgroups excluded (coding scheme A6 in Table 2 of the main text). Pie charts at nodes and tips represent the probabilities of the inferred chromosome number(s); numbers inside charts have the highest probability. The numbers at tips are the input chromosome numbers used in the 'phylogenetically informed' coding scheme (see Materials and Methods). Numbers above branches represent the inferred frequency of those of the four possible events (gains, losses, duplications, demi-duplications) that had a posterior probability >0.5. The colour-coding of chromosome numbers and the four events is explained in the insets.

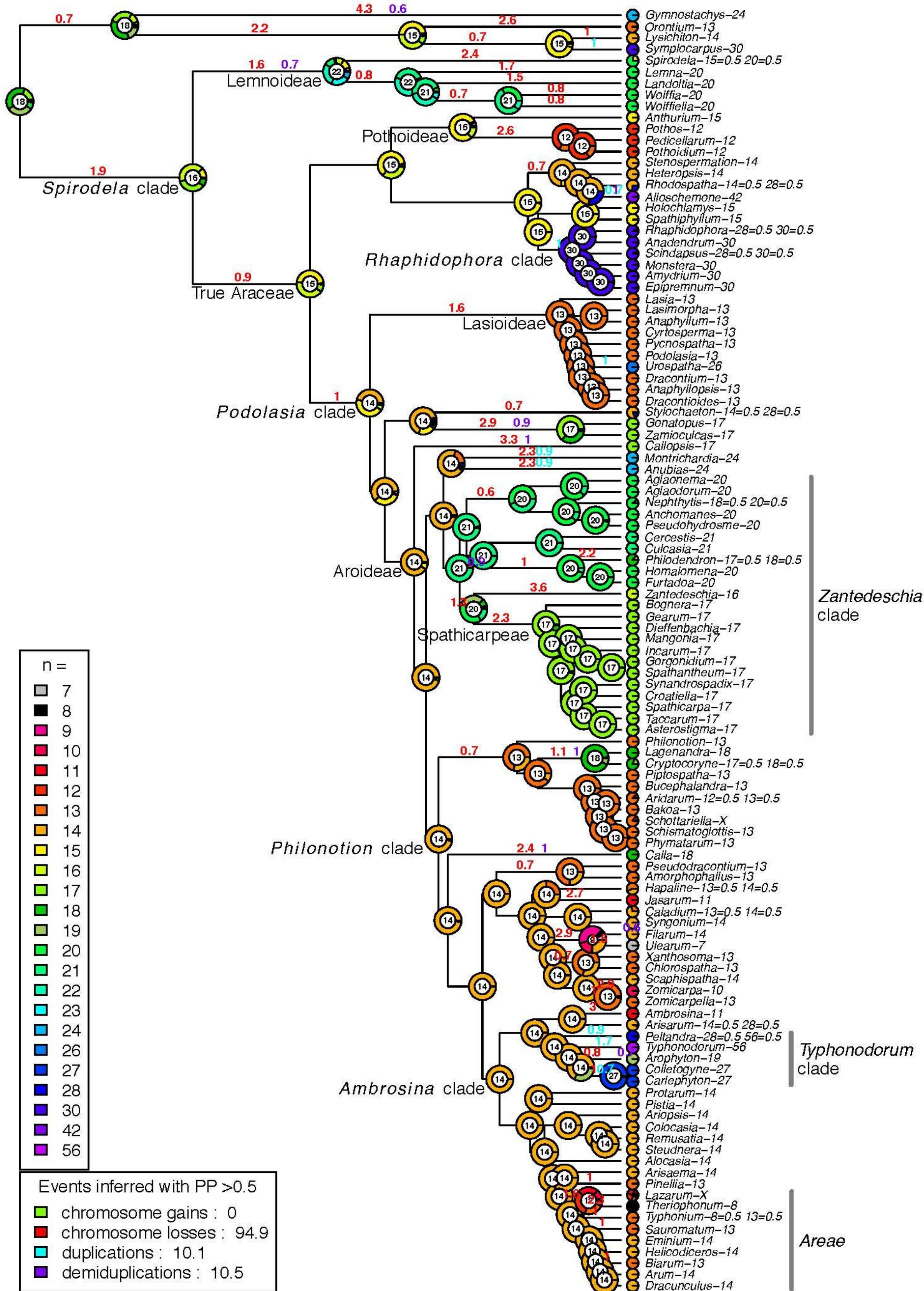
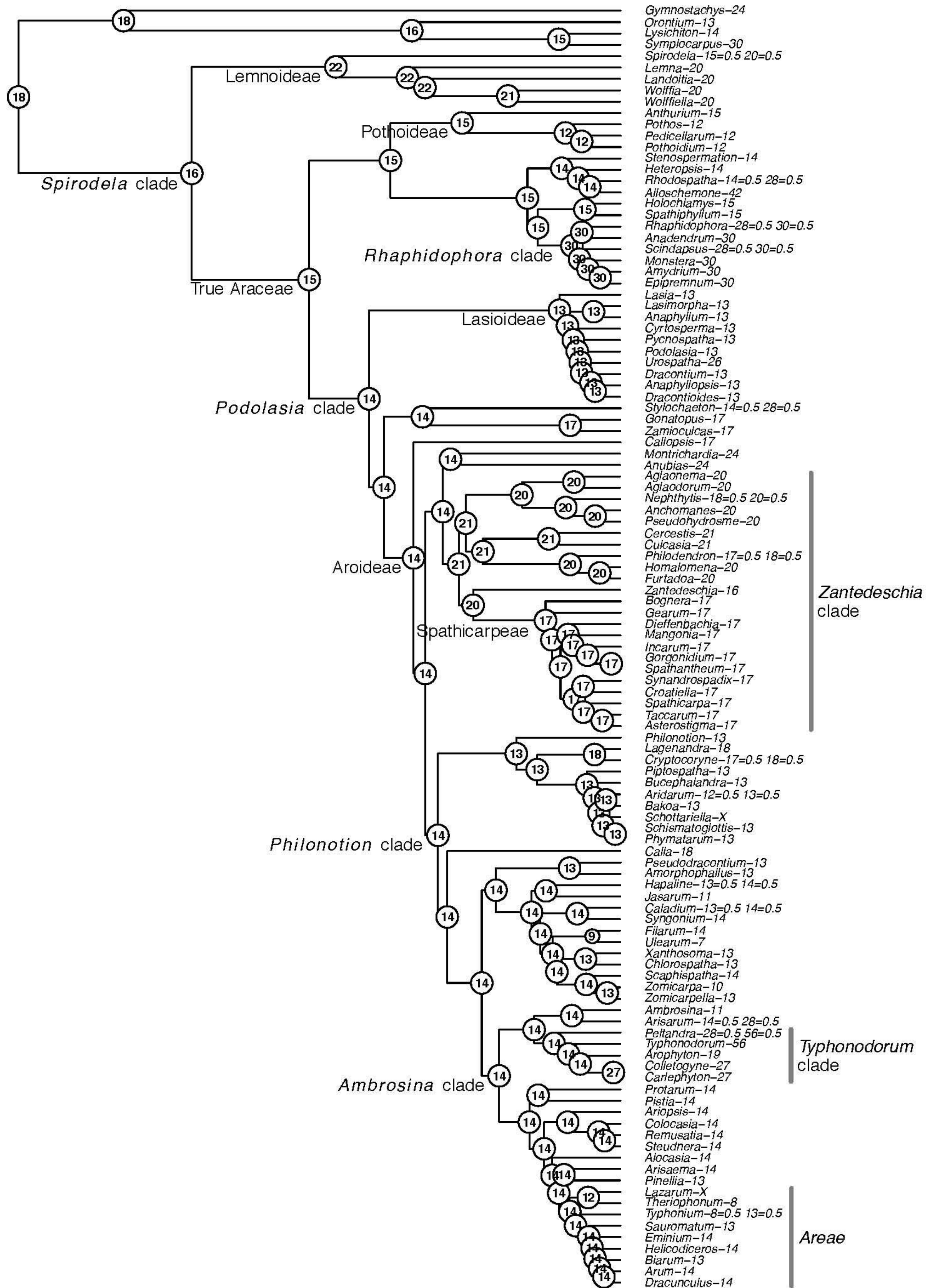


FIG. S2. Chromosome number evolution in Araceae inferred under maximum likelihood optimization, with phylogenetically informed coding and outgroups excluded (analysis A6 in Table 2).



SUPPLEMENTARY DATA

TABLE S1. Chromosome counts for species of Araceae with references, including the four new counts reported in the present paper. Where a species' name has been changed due to taxonomic revision, the name under which the number was originally published is given in brackets.

Species	*n*	2*n*	References
Aglaodorum griffithii		40	Petersen, 1989
Aglaonema commutatum		14	Subramanian & Munian, 1988
Aglaonema modestum	20 II + 20I		Srivastava, 1982
Aglaonema oblongifolium		40	Marchant, 1971a
Aglaonema pictum		40	Okada, 1982
Aglaonema simplex		40	Marchant, 1971a
Aglaonema treubii		100	Marchant, 1971a
Alloschemone occidentalis		*84*	Bogner & Petersen, 2007
Alocasia acuminata		28	Mehra & Sachdeva 1979 Petersen, 1989
Alocasia alba (Alocasia crassifolia)		28	Okada, 1982 Petersen, 1989
Alocasia argyrea not accepted		56	Sharma, 1970
Alocasia brancifolia		28	Petersen, 1989
Alocasia cucullata		28	Ankei, 1987; Petersen, 1989
Alocasia cuprea	14	28	Petersen, 1989
Alocasia decipiens		24, 28	Bhattacharya, 1974 Petersen, 1989
Alocasia fornicata	14	28, 42	Ramachandran, 1978 Petersen, 1989
Alocasia lauterbachiana (Xenophya lauterbachiana)		28	Petersen, 1989
Alocasia lauterbachiana (Alocasia wavriniana)		28	Marchant, 1971a
Alocasia longiloba		28, 56	Sharma, 1970 Marchant, 1971a
Alocasia longiloba var. denudata (Alocasia denudata)		28	Marchant, 1971a
Alocasia longiloba var. korthalsii (Alocasia korthalsii)		28	Petersen, 1989
Alocasia longiloba var. lowii (Alocasia lowii)		28, 40	Bhattacharya, 1974 Petersen, 1989
Alocasia longiloba var. putzeysii (Alocasia putzeysii)		70, 84	Petersen, 1989
Alocasia longiloba var. thibautiana (Alocasia		28	Sharma, 1970

thibautiana)			
Alocasia macrorrhiza		26, 28	Sharma, 1970 Petersen, 1989
Alocasia macrorrhizos (Alocasia indica)	21	28, 42	Ramachandran, 1978 Bhattacharya, 1974 Petersen, 1989
Alocasia microlitziana		28	Petersen, 1989
Alocasia Montana not accepted		28	Ramachandran, 1978
Alocasia navicularis		28, 68	Sharma, 1970 Petersen, 1989
Alocasia odora		28, 56	Nguyen et al., 1998 Petersen, 1989
Alocasia putii		28	Sharma, 1970
Alocasia regina		28	Sharma, 1970
Alocasia sanderiana	14		Petersen, 1989
Alocasia zebrina		28, 42	Sharma, 1970; Bhattacharya, 1974
Ambrosina bassii		22	Petersen, 1989
Amorphophallus abyssinicus		26	Chauhan & Brandham, 1985
Amorphophallus albus		26	Liu et al., 1985
Amorphophallus ankarana		26	Hetterscheid et al., 1999
Amorphophallus asterostigmatus		26	Petersen, 1992
Amorphophallus bannanensis		26	Gu et al., 1992
Amorphophallus bulbifer		26, 39	Chauhan & Brandham, 1985 Kuruvilla et al., 1989
Amorphophallus campanulatus	14	26, 28	Sharma, 1970 Chaudhuri & Sharma, 1979
Amorphophallus commutatus		26	Chauhan & Brandham, 1985
Amorphophallus dixenii		28	Larsen & Larsen, 1974
Amorphophallus dracontioides		26	Chauhan & Brandham, 1985
Amorphophallus dubius		28	Chauhan & Brandham, 1985
Amorphophallus dunnii		26	Zheng & Liu, 1989
Amorphophallus eichleri		26 (56)	Petersen, 1989
Amorphophallus gallaënsis		26	Petersen, 1989
Amorphophallus giganteus		39	Petersen, 1989
Amorphophallus goetzii		26	Chauhan & Brandham, 1985
Amorphophallus gombocziamus		26	Marchant, 1971b
Amorphophallus hildebrandtii		26	Chauhan & Brandham, 1985
Amorphophallus hirtus		26	Petersen, 1989
Amorphophallus hohenackeri		26	Petersen, 1989

Amorphophallus johnsonii		26	Chauhan & Brandham, 1985
Amorphophallus kerrii		26	Chauhan & Brandham, 1985
Amorphophallus konjac		26, 39	Ishida & Akagi, 2000 Petersen, 1989
Amorphophallus konkanensis		26	Patil & Dixit, 1995
Amorphophallus lambii		26	Chauhan & Brandham, 1985
Amorphophallus laxiflorus		26	Chauhan & Brandham, 1985
Amorphophallus linumaana		26	Petersen, 1989
Amorphophallus longituberosus		28 (56)	Petersen, 1989
Amorphophallus maculatus		26	Petersen, 1989
Amorphophallus mairei		26	Zheng & Liu, 1989
Amorphophallus margaritifer		26	De Sarker & Hetterscheid, 1997
Amorphophallus maximus		26	Petersen, 1989
Amorphophallus napalensis		28	Petersen, 1989
Amorphophallus oncophyllus		39	Chauhan & Brandham, 1985
Amorphophallus paeoniifolius	14	26, 28	Chauhan & Brandham, 1985 Petersen, 1989
Amorphophallus palawanensis		26	Petersen, 1992
Amorphophallus prainii		28	Chauhan & Brandham, 1985
Amorphophallus rivieri		26	Zheng & Liu 1989
Amorphophallus siamensis		26	Petersen, 1989
Amorphophallus sinensis		26	Sun, 1999
Amorphophallus sumawongii		26	Petersen, 1989
Amorphophallus sutepensis		26	Chauhan & Brandham, 1985
Amorphophallus sylvaticus		26	Petersen, 1989
Amorphophallus taurostigma		26	Hetterscheid et al., 1999
Amorphophallus thomsonii		26 (24, 25)	Petersen, 1989
Amorphophallus titanum	13	26	Giordano, C. 1999 Petersen, 1989
Amorphophallus variabilis	13	26	Chauhan & Brandham, 1985 Petersen, 1989
Amydrium humile		60	Petersen, 1989
Amydrium medium		60	Petersen, 1989
Anadendrum marginatum		60	Petersen, 1989
Anadendrum		60	Petersen, 1989

microstachyum			
Anadendrum montanum		60	Petersen, 1989
Anaphyllopsis americana		26	Petersen, 1989
Anaphyllum beddomei		Ca. 26	Petersen, 1989
Anaphyllum wightii	13	26	Ramachandran, 1978 Petersen, 1989
Anaphyllum wightii ssp. revolutum		26	Petersen, 1989
Anchomanes abbreviatus		40	Petersen, 1989
Anchomanes difformis		40	Petersen, 1989
Anchomanes welwitchii		40	Marchant, 1971a
Anthurium acaule	15	30 + 2B, 0-2B or 2-5B	Sharma, 1970 Bhattacharya, 1976 Vij et al., 1982
Anthurium acussatum	c. 15	c. 30	Petersen, 1989
Anthurium acutangulum	c. 15	30	Sheffer & Croat, 1983 Petersen, 1989
Anthurium acutum		30	Petersen, 1989
Anthurium aemulum		30, 60	Sheffer & Kamemoto, 1976
Anthurium affine		30	Carvalheira et al., 1991
Anthurium allenii		30	Sheffer & Kamemoto, 1976
Anthurium amnicola		30	Marutani et al., 1988
Anthurium andicola		30	Sheffer & Croat, 1983
Anthurium andraeanum	c. 15, 16	30, 32	Sheffer & Croat, 1983 Petersen, 1989
Anthurium angustispadix		30	Sheffer & Croat, 1983
Anthurium antioquiense		30	Sheffer & Croat, 1983
Anthurium armeniense		30	Sheffer & Croat, 1983
Anthurium aureum		30, 31	Sheffer & Kamemoto, 1976
Anthurium baileyi		60	Sheffer & Kamemoto, 1976
Anthurium bakeri	15	30	Sheffer & Croat, 1983 Petersen, 1989
Anthurium bellum		28, 56	Bhattacharya, 1976 Vij et al., 1982 Petersen, 1989
Anthurium beltianum		30	Sheffer & Croat, 1983
Anthurium berriozabalense		30	Sheffer & Croat, 1983
Anthurium bicollectivum		28, 30	Sheffer & Croat, 1983
Anthurium boucheanum		56	Petersen, 1989
Anthurium brenesii		30	Sheffer & Croat, 1983
Anthurium bristanii		30	Petersen, 1989
Anthurium brittonianum		30	Sheffer & Croat, 1983
Anthurium brownii		30	Sheffer & Croat, 1983
Anthurium caperatum		30	Marutani et al. 1993
Anthurium cerrobaulense		30	Sheffer & Croat, 1983
Anthurium cerrocampanense		30, 30 + 2B	Sheffer & Croat, 1983 Marutani et al., 1993
Anthurium chamulense		30	Sheffer & Croat, 1983

Anthurium chiriquense		30	Sheffer & Kamemoto, 1976
Anthurium circinatum		30	Sheffer & Croat, 1983
Anthurium clarinervium	15	30	Sheffer & Croat, 1983
			Sheffer and Croat, 1983
Anthurium clavatum		30	Sheffer & Croat, 1983
Anthurium clavigerum		30	Petersen, 1989
Anthurium clidemioides		30	Petersen, 1989
Anthurium colonicum		30	Sheffer & Croat, 1983
Anthurium comtum	15	c. 30	Petersen, 1989
Anthurium concinnatum		30	Sheffer & Kamemoto, 1976
Anthurium concolor		30	Sheffer & Croat, 1983
Anthurium consobrinum	15	30	Sheffer & Croat, 1983
			Petersen, 1989
Anthurium cordatum	c. 15	c. 30	Petersen, 1989
			Sheffer & Croat, 1983
Anthurium cotobrusii		60	Sheffer & Croat, 1983
Anthurium crassinervium	c. 30	60	Sheffer & Croat, 1983
			Petersen, 1989
Anthurium crassiradicans		30	Petersen, 1989
Anthurium crenatum	15	30	Petersen, 1989
Anthurium crystallinum	15	30 +0-3B	Bhattacharya, 1976
			Vij et al., 1982
			Sharma, 1970
Anthurium cubense	c. 15	30	Sheffer & Croat, 1983
			Petersen, 1989
Anthurium cucullispathum		30	Sheffer & Croat, 1983
Anthurium curvilaminum		30	Sheffer & Croat, 1983
Anthurium cuspidatum		30	Sheffer & Croat, 1983
Anthurium denudatum	c. 15	30	Sheffer & Kamemoto, 1976
			Petersen, 1989
Anthurium digitatum	30	26, 30, 36	Sheffer & Kamemoto, 1976
			Bhattacharya, 1976
			Vij et al., 1982
			Sharma, 1970
			Petersen, 1989
Anthurium dominicense	15	c. 30	Petersen, 1989
Anthurium durandii	15	c. 30	Petersen, 1989
Anthurium ellipticum		30	Sheffer & Kamemoto, 1976
Anthurium fatoense		Ca. 30	Sheffer & Croat, 1983
Anthurium flavoviride		30	Sheffer & Kamemoto, 1976
Anthurium flexile		60	Sheffer & Croat, 1983
Anthurium folsonii		30	Petersen, 1989
Anthurium forgetii	15	30 + Bs	Sheffer & Kamemoto, 1976
			Petersen, 1989
			Sheffer & Croat, 1983
Anthurium formosum		30	Marutani et al., 1993
Anthurium friedrichsthalii	15	30	Sheffer & Kamemoto, 1976
			Petersen, 1989
Anthurium garagaranum		30 + 0-1B	Marutani et al., 1993

Anthurium gladiifolium		30	Sheffer & Kamemoto, 1976
Anthurium gracile		20, 30, 40, 49, 60	Sheffer & Croat, 1983 Guerra, 1986 Sheffer & Kamemoto, 1976 Petersen, 1989
Anthurium grande		28, 30	Sheffer & Kamemoto, 1976 Sharma, 1970
Anthurium grandifolium		30	Sheffer & Kamemoto, 1976
Anthurium gustavii		30	Sheffer & Kamemoto, 1976
Anthurium gymnopus		30	Petersen, 1989
Anthurium hacumense		30	Sheffer & Croat, 1983
Anthurium harrisii		28 + 2B, 30	Bhattacharya, 1976 Vij et al., 1982 Petersen, 1989
Anthurium hoffmannii		30	Sheffer & Kamemoto, 1976
Anthurium hookeri	15	30, 60	Sheffer & Kamemoto, 1976 Petersen, 1989
Anthurium hornitense		30	Sheffer & Croat, 1983
Anthurium huixtlense		30	Sheffer & Croat, 1983
Anthurium hutchisonii		30	Sheffer & Croat, 1983
Anthurium imperiale		30 + f, 60	Marchant, 1973 Petersen, 1989
Anthurium jenmanii		48	Sheffer & Croat, 1983
Anthurium joseanum		30	Sheffer & Kamemoto, 1976
Anthurium kamemotoanum		30	Marutani et al., 1993
Anthurium lancifolium		30	Sheffer & Croat, 1983
Anthurium lentii		30	Sheffer & Croat, 1983
Anthurium leuconeurum		35	Sheffer & Croat, 1983
Anthurium lezamae		30	Sheffer & Croat, 1983
Anthurium lindenianum		30	Marutani et al., 1993
Anthurium lindenianum		30	Sheffer & Kamemoto, 1976
Anthurium littorale		28	Sheffer & Kamemoto, 1976
Anthurium longipeltatum		30	Sheffer & Croat, 1983
Anthurium longistipitatum		30	Sheffer & Croat, 1983
Anthurium lucens		30, 66	Sheffer & Croat, 1983
Anthurium lucidum		c. 124	Petersen, 1989
Anthurium luteynii		30	Sheffer & Croat, 1983
Anthurium magnificum	c. 15	30, 60	Sheffer & Croat, 1983 Sheffer & Kamemoto, 1976 Petersen, 1989
Anthurium maximum	c. 15	c. 30	Petersen, 1989
Anthurium mexicanum		60	Sheffer & Kamemoto, 1976
Anthurium michelii		30	Sheffer & Croat, 1983
Anthurium micromystrium		30	Sheffer & Kamemoto, 1976
Anthurium microphyllum		30 + B	Petersen, 1989
Anthurium microspadix		c. 30, 60	Sheffer & Croat, 1983 Petersen, 1989
Anthurium montanum		30	Sheffer & Croat, 1983
Anthurium nervatum		30	Sheffer & Croat, 1983

Anthurium nymphaeifolium		28, 30	Marutani et al., 1993 Bhattacharya, 1976 Vij et al., 1982
Anthurium obtusilobum		30	Sheffer & Croat, 1983
Anthurium ochranthum		30 + 2B	Marutani et al., 1993
Anthurium oerstedianum		30	Sheffer & Croat, 1983
Anthurium olfersianum	c. 15	30 + B	Petersen, 1989 Sheffer & Croat, 1983
Anthurium ovandense		30	Sheffer & Croat, 1983
Anthurium paludosum		30	Petersen, 1989
Anthurium papillaminum		30	Petersen, 1989
Anthurium paraguayense		60	Fernandez, A. 1977
Anthurium patulum	14	28 + B	Petersen, 1989 Sheffer & Croat, 1983
Anthurium pedatoradiatum	c. 15	c. 30	Petersen, 1989
Anthurium pentaphyllum	15	60	Sheffer & Kamemoto, 1976 Petersen, 1989
Anthurium pichinchae		30	Sheffer & Kamemoto, 1976
Anthurium pittieri		30	Sheffer & Croat, 1983
Anthurium pluricostatum		30	Sheffer & Croat, 1983
Anthurium podophyllum	15	30	Bhattacharya, 1976 Vij et al., 1982
Anthurium procerum		30	Sheffer & Kamemoto, 1976
Anthurium pseudospectabile		30	Sheffer & Croat, 1983
Anthurium pulchellum		63	Petersen, 1989
Anthurium purpureospathum		30	Sheffer & Croat, 1983
Anthurium radicans	15	30	Sheffer & Croat, 1983 Petersen, 1989
Anthurium ramonense		30	Sheffer & Croat, 1983
Anthurium ranchoanum		30	Sheffer & Kamemoto, 1976
Anthurium ravenii		30	Sheffer & Croat, 1983
Anthurium regale		30 + 1B	Sheffer & Kamemoto, 1976
Anthurium rhodostachyum		28, 29, 30, 31	Sheffer & Kamemoto, 1976
Anthurium roraimense		30	Sheffer & Kamemoto, 1976
Anthurium roseospadix		30	Marutani et al., 1993
Anthurium rzedowskii		30	Sheffer & Croat, 1983
Anthurium sagawanae		30	Sheffer & Croat, 1983
Anthurium salvadorense		30	Sheffer & Croat, 1983
Anthurium salviniae		30	Sheffer & Croat, 1983
Anthurium sanctifidense		30	Marutani et al., 1993
Anthurium scandens	16, 24	24, 48, 84	Sheffer & Kamemoto, 1976 Sheffer & Croat, 1983
Anthurium scandens. scandens		48, 84	Sheffer & Croat, 1983 Petersen, 1989
Anthurium scherzerianum	15, 16	14, 30, 32	Subramanian & Munian, 1988 Sheffer & Croat, 1983

			Petersen, 1989
Anthurium schlechtendalii	15	30	Sheffer & Croat, 1983
Anthurium schottianum		30	Sheffer & Croat, 1983
Anthurium scolopendrinum		20, 40	Sheffer & Kamemoto, 1976
Anthurium seibertii		30	Sheffer & Croat, 1983
Anthurium seleri		30	Sheffer & Croat, 1983
Anthurium sellowianum	15		Petersen, 1989
Anthurium signatum		30 + B, 34	Sheffer & Croat, 1983 Petersen, 1989
Anthurium solitarum		30 + B, 34	Sheffer & Croat, 1983 Petersen, 1989
Anthurium splendidum	15	30 + Bs	Bhattacharya, 1976 Vij et al., 1982 Sharma, 1970
Anthurium standleyi		60	Sheffer & Croat, 1983
Anthurium subhastatum		30	Sheffer & Kamemoto, 1976
Anthurium subovatum		30	Sheffer & Croat, 1983
Anthurium subsignatum		30	Marutani et al., 1993
Anthurium supianum		c. 90	Sheffer & Kamemoto, 1976
Anthurium tenerum		30	Sheffer & Croat, 1983
Anthurium testaceum		30	Sheffer & Croat, 1983
Anthurium tonduzii		30	Sheffer & Croat, 1983
Anthurium trianae		28, 29 + 1B	Sheffer & Kamemoto, 1976
Anthurium triangulum		30	Sheffer & Kamemoto, 1976
Anthurium trinerve		24, 30	Petersen, 1989
Anthurium trinerve		24, 30	Sheffer & Croat, 1983
Anthurium triphyllum	30	60	Bhattacharya, 1976 Vij et al., 1982
Anthurium turrialbense		30	Sheffer & Kamemoto, 1976
Anthurium umbrosum		30	Sheffer & Croat, 1983
Anthurium undatum		c. 60 + B	Marchant, 1973
Anthurium upalaense		30	Sheffer & Croat, 1983
Anthurium vallense		30	Sheffer & Croat, 1983
Anthurium veitchii	15	30	Sheffer & Kamemoto, 1976
Anthurium velutium		30	Sheffer & Kamemoto, 1976
Anthurium venosum		30	Sheffer & Kamemoto, 1976
Anthurium wallisii		30 + 2B, c. 60	Sheffer & Kamemoto, 1976
Anthurium warocqueanum	15	30 + Bs	Marutani & Kamemoto, 1983 Petersen, 1989
Anthurium watermaliense		30	Sheffer & Croat, 1983
Anthurium wendlingeri		30	Sheffer & Croat, 1983
Anthurium wullschlaegelii		30	Sheffer & Kamemoto, 1976
Anubias afzelii		48	Arends & van der Laan, 1982
Anubias gigantea		48	Arends & van der Laan, 1982
Anubias gilletii		48	Arends & van der Laan, 1982

Anubias gracilis	48	Arends & van der Laan, 1982
Anubias hastifolia	48	Arends & van der Laan, 1982
Anubias heterophylla	48	Arends & van der Laan, 1982
Anubias lanceolata	48	Marchant, 1971a
Anubias pynaertii	48	Arends & van der Laan, 1982
Apoballis (Schismatoglottis) belophylla	26	Petersen, 1989
Apoballis (Schismatoglottis) brevipes	26	Okada, 2000
Apoballis (Schismatoglottis) okadae	26 + Bs	Okada, 2000 Petersen, 1989
Apoballis (Schismatoglottis) rupestris	26 + B	Okada, 1982
Apoballis acuminatissima (Schismatoglottis concinna)	26 + B	Marchant, 1971a
Apoballis acuminatissima (Schismatoglottis kurimana)	26, 39	Petersen, 1989
Apoballis mutata (Schismatoglottis batoeensis)	26	Okada, 1982
Apoballis rupestris (Schismatoglottis treubii)	26	Petersen, 1989
Apoballis rupestris (Schismatoglottis wigmannii)	56	Petersen, 1989
Aridarum annae	24	Petersen, 1989
Aridarum burttii	26	Okada, 2000
Aridarum incavum	26	Okada, 2000
Aridarum nicolsonii	24, 26	Okada, 2000 Petersen, 1989
Ariopsis peltata	28, 84, 86	Petersen, 1989 Marchant, 1971a
Arisaema aequinoctiale	26	Watanabe et al., 1998
Arisaema album	28	Petersen, 1989
Arisaema amurense	26,28, 39, 48, 52, 56, 70	Ae, 1975 Serizawa, 1981 Murata, 1990 Sokolovskaya & Probatova, 1985 Petersen, 1989
Arisaema angustatum	28	Watanabe et al., 1998
Arisaema aprile	28	Murata, J. 1983
Arisaema atrorubens	56	Kapoor, B. M. 1982
Arisaema biauriculatum	28	Gu, Z.-j. & H. Sun 1998
Arisaema candidissimum	56	Petersen, 1989
Arisaema caudatum	28	Patil, K. S. & G. B. Dixit 1995

Arisaema concinum		28, 56	Mehra, P. N. & S. K. Sachdeva 1976
			Petersen, 1989
Arisaema consanguineum		28, 48,	Wang, Jenn-che 1996
		56	Sarkar, A. K. & N. Datta 1978
Arisaema consenouinum		28	Sarkar, A. K. 1991
Arisaema costatum		20	Murata, J. 1990
Arisaema cucullatum		28	Petersen, 1989
Arisaema curvatum		28	Mehra, P. N. & S. K. Sachdeva 1976
Arisaema decipiens		28	Sharma, 1970
Arisaema dracontium		56	Murata, J. & M. Iijima 1983
Arisaema dulongense		26	Gu, Z.-j., L. Wang & H. Li 1992
Arisaema echinatum		28	Petersen, 1989
Arisaema ehimense		28	Murata, J. & J. Ohno 1989
Arisaema erubescens		28, 56	Gu, Z.-j., L. Wang & H. Li 1992
			Mehra, P. N. & S. K. Sachdeva 1976
Arisaema filiforme		28	Murata, J. 1990
Arisaema flavum		56	Murata, J. & M. Iijima 1983
Arisaema formosanum		28, 56	Wang, Jenn-che 1996
Arisaema franchetianum		56	Murata, J. & M. Iijima 1983
Arisaema galeatum		26	Murata, J. 1990
Arisaema grapsospadix		28	Wang, Jenn-che 1996
Arisaema griffithii		28, 32	Sharma, 1970
		+ 1B	Bhattacharya, 1978
Arisaema hatizyoense		26	Watanabe et al., 1998
Arisaema heterocephalum		28	Petersen, 1989
Arisaema heterophyllum		28, 56,	Murata, 1990
		64, 84,	Wang, 1996
		140,	Ko & Kim, 1985
		168	
Arisaema ilanense		28	Wang, 1996
Arisaema inclusum		24	Murata & Iijima, 1983
Arisaema intermedium	14	28	Mehra & Sachdeva, 1976
			Sachdeva, 1977
Arisaema iyonum		28	Petersen, 1989
Arisaema jacquemontii		28, 52	Mehra & Sachdeva, 1976
			Petersen, 1989
Arisaema japonicum		26, 28,	Watanabe et al., 1998
		42	Petersen, 1989
Arisaema kawashimae		28	Serizawa, 1980
Arisaema kelung-insulares		28	Petersen, 1989
Arisaema kishidae		28	Watanabe et al., 1998
Arisaema kiushianum		56	Petersen, 1989
Arisaema komarovii		56	Sokolovskaya & Probatova, 1985
Arisaema leschenaultii		28	Petersen, 1989
Arisaema limbatum		26	Watanabe et al., 1998
Arisaema lobatum		28, 56,	Murata, 1990
		Ca. 84	Hong & Zhang, 1990
Arisaema longilaminum		28	Petersen, 1989
Arisaema longipedunculatum		28, 56	Serizawa, 1981

Arisaema macrospathum		28	Pringle, 1979
Arisaema maximowiczii		28	Petersen, 1989
Arisaema minamitanii		28	Murata, 1990
Arisaema minus		26	Watanabe et al., 1998
Arisaema monophyllum		28	Murata &. Iijima, 1983
Arisaema murrayi	14	28, 56	Patil & Dixit, 1995
Arisaema nambae		28	Watanabe et al., 1998
Arisaema nanjenense		28	Huang & Wu, 1997
Arisaema negishii		28	Ko et al., 1987
Arisaema neglectum		28, 56	Ramachandran, 1978
			Subramanian & Munian, 1988
Arisaema nepenthoides		26 + 1B	Bhattacharya, 1978
Arisaema nikoense		28	Watanabe et al., 1998
Arisaema ogatae		28	Petersen, 1989
Arisaema ostiolatum		28	Petersen, 1989
Arisaema ovale		26, 52,	Ko et al., 1987
		56, 112	Petersen, 1989
Arisaema peninsulae		26, 28	Lee, 1967
Arisaema pingbianense		28	Murata, 1990
Arisaema polyphyllum		28	Petersen, 1989
Arisaema propiquum		28	Petersen, 1989
Arisaema rhizomatum		28	Murata & Iijima 1983
Arisaema ringens		28, 56	Watanabe et al., 1998
			Petersen, 1989
Arisaema robustum		56	Ko & Kim 1985
Arisaema roxburghii		24	Petersen, 1989
Arisaema sachalinense		56	Murata, J. 1990
Arisaema sahyadricum		28	Patil & Dixit 1995
Arisaema sanguineum		28	Sharma & Mukhopadhyay, 1963
Arisaema sazenzo		28	Murata & Iijima, 1983
Arisaema schimperianum		28	Petersen, 1989
Arisaema seppikoense		26	Watanabe et al., 1998
Arisaema serratum		26, 28	Lijima, 1982
Arisaema sikkimense		26 + 1B	Bhattacharya, 1978
Arisaema sikokianum		28	Watanabe et al., 1998
Arisaema speciosum		28	Sharma, 1970
Arisaema stenophyllum		26	Watanabe et al., 1998
Arisaema taiwanense		28	Wang, Jenn-che 1996
Arisaema takedae		28	Petersen, 1989
Arisaema takesimense		28	Ko et al., 1987
Arisaema tashiroi		28	Petersen, 1989
Arisaema ternatipartitum		72	Watanabe et al., 1998
Arisaema thunbergii		28	Ko & Kim, 1985
Arisaema tortuosum	14, 26	24, 26,	Mehra & Sachdeva, 1976
		28, 56	Ramachandran, 1978
			Sachdeva, 1977
			Sharma & Mukhopadhyay, 1963
Arisaema tosaense		28	Watanabe et al., 1998
Arisaema triphyllum		28, 56	Hill, 1995
			Sachdeva, 1977

Arisaema undulatifolium		26	Watanabe et al., 1998
Arisaema urashima		28	Watanabe et al., 1998
Arisaema wallichianum	14	26 + 4B	Mehra & Sachdeva, 1976 Sharma, 1970
Arisaema wightianum		28	Subramanian & Munian, 1988
Arisaema wightii		28	Ramachandran, 1978
Arisaema yamatense		28	Watanabe et al., 1998
Arisaema yunnanense		48	Murata & Iijima, 1983
Arisarum proboscideum		14, 28, 42, 56	Diosdado et al., 1993 Petersen, 1989
Arisarum vulgare	28	56	Aboucaya & Verlaque, 1990 Petersen, 1989
Arophyton buchetii		40	Petersen, 1989
Arophyton crassifolium		54	Petersen, 1989
Arophyton humbertii		38	Petersen, 1989
Arophyton rhizomatosum		38	Petersen, 1989
Arophyton simplex		38	Sharma, 1970
Arophyton tripartitum		c. 76	Marchant, 1970
Arum alpinum = Arum cylindraceum		28	D'Emerico et al., 1993
Arum apulum		56, 63, 70	Bianco et al., 1993
Arum arisarum		56	Fernandez & Ruiz Rujon, 1976
Arum byzantinum		28	Alpinar, 1986
Arum concinnatum		84	Alpinar, 1986
Arum creticum		28	Alpinar, 1987
Arum cyrenaicum		56	Petersen, 1989
Arum detruncatum		28	Alpinar, 1986
Arum dioscoridis		28	Alpinar, 1986
Arum elongatum		28	Alpinar, 1986
Arum euxinum		28	Alpinar, 1986
Arum hygrophilum		28, 29	Bedalov, 1978 Petersen, 1989
Arum idaeum		28	Bedalov & Küpfer, 2006
Arum italicum		28, 70, 84	Alpinar, 1986 Petersen, 1989
Arum korolkowii		28	Petersen, 1989
Arum maculatum		28, 30, 42, 56	Šopova & Sekovski, 1989 Mesíček, 1992 D'Emerico et al., 1993 Petersen, 1989
Arum nickelii		84	Alpinar, 1986
Arum nigrum		28	D'Emerico et al., 1993
Arum orientale		28	Bedalov et al., 1998
Arum palaestinum		28	Bedalov, 1978
Arum petteri		28	Petersen, 1989
Arum pictum		28	D'Emerico et al., 1993
Arum purpureospathum		56	Bedalov & Küpfer, 2006
Arum rupicola		28	Bedalov & Küpfer, 2006
Arum sintenisii		28	Bedalov & Küpfer, 2006

Arum sooi		42	Bedalov & Terpo, 1998
Asterostigma cryptostylum		34	Bogner, 1997
Asterostigma lividum		34 + Bs	Petersen, 1989
Bakoa (Piptospatha) brevipedunculata		26	Okada, 2000
Bakoa (Piptospatha) lucens		26	Okada, 2000
Biarum bovei		74	Petersen, 1989
Biarum carduchorum		24	Petersen, 1989
Biarum carratracense		22, 36,	Fernandez Piqueras & Ruiz Rujon, 1976
		c. 96,	Fernandez et al., 1978
		98	Marchant, 1971b
Biarum davisii		26	Petersen, 1989
Biarum dispar		74	Talavera, 1976
Biarum ditschianum		26	Petersen, 1989
Biarum eximium		16	Petersen, 1989
Biarum fraasianum		32	Popova & Ceschmedjiev, 1978
Biarum kotschyi		c. 96	Petersen, 1989
Biarum marmarisense		22, 24,	Athanasiou & Kamari, 1992
		26	Gill, 1988
Biarum pyrami		108	Borzatti von Löwenstern & Garbari, 1999
Biarum tenuifolium		16, 18,	Athanasiou & Kamari, 1992
		26	Petersen, 1989
Biarum tenuifolium (B. spruneri)		26, 40	Athanasiou & Kamari, 1992
Biarum tenuifolium ssp. arundanum (Biarum arundanum)		22	Elena Rossello & Gallego, 1984
Biarum tenuifolium ssp. galiani (Biarum galiani)		26	Elena Rossello & Gallego, 1984
Bognera recondita		34	Bogner, 2008
Bucephalandra catherineae		26	Okada, 2000
Bucephalandra magnifolia		26	Okada, 2000
Bucephalandra motleyana		26	Okada, 2000
Caladium bicolor	15	22, 26,	Ramachandran, 1978
		28, 30	Sarkar, 1975
			Sarkar, 1976
Caladium chanjur		28	Petersen, 1989
Caladium humboldtii		19	Petersen, 1989
Caladium lindenii	13		Petersen, 1989
Caladium macrotites		30	Petersen, 1989
Caladium striatipes		22	Petersen, 1989
Calla palustris	18, 36	36, 60,	Uotila & Pellinen, 1985
		72	Kartashova et al., 1974
			Geber & Schweizer, 1988
			Petersen, 1989
Callopsis volkensii		36	Marchant, 1971a
Carlephyton diegoense		c. 108	Petersen, 1989
Carlephyton		54	Marchant, 1973

glaucophyllum			Petersen, 1989
Carlephyton madagascariense		108	Marchant, 1970
Cercestis afzelii		42	Petersen, 1989
Cercestis camerunensis		c. 42	Petersen, 1989
Cercestis mirabilis		42	Petersen, 1989
Cercestis sagittatus		42	Petersen, 1989
Cercestis stigntaticus		c. 36	Petersen, 1989
Cercestis talensis		42	Petersen, 1989
Chlorospatha corrugata		26	Bogner, 1985
Chlorospatha longipoda		26	Petersen, 1989
Colletogyne perrieri		44, 46, 54	Sharma, 1970 Marchant, 1973 Petersen, 1989
Colocasia affins		28	Petersen, 1989
Colocasia antiquorum	14	26, 28, 30, 36, 38, 42, 44, 46, 48, 52, 58, 116	Subramanian, 1979 Sarkar, 1991 Subramanian & Munian, 1988 Chaudhuri & Sharma, 1979
Colocasia esculenta	14	28, 36, 38, 42, 48, 84	Ramachandran, 1978 Tanimoto & Matsumoto, 1986 Huang et al., 1989 Sreekumari & Mathew, 1991 Subramanian & Munian, 1988
Colocasia gigantea	14	28, 42	Tanimoto & Matsumoto, 1986 Petersen, 1989
Colocasia indica		28	Ankei, 1987
Croatiella integrifolia		34	Bogner, 2008
Cryptocoryne affinis		34	Arends et al., 1982
Cryptocoryne albida		36	Arends et al., 1982
Cryptocoryne amicorum		34	Arends et al., 1982
Cryptocoryne annamica		34	Petersen, 1993
Cryptocoryne aponogetifolia		34	Petersen, 1989
Cryptocoryne auriculata		34	Petersen, 1993
Cryptocoryne balansae		36	Jacobson, 1977
Cryptocoryne beckettii		28, 42	Arends et al., 1982; Petersen, 1989
Cryptocoryne bertelibansenii		36	Jacobson, 1977
Cryptocoryne blassii		102	Jacobson, 1977
Cryptocoryne bogneri		36	Jacobson, 1977
Cryptocoryne bullosa		34	Jacobson, 1977
Cryptocoryne ciliata		22, 33	Jacobson, 1977
Cryptocoryne cognata		28	Petersen, 1993a
Cryptocoryne consobrina		36	Petersen, 1989
Cryptocoryne cordata		28, 34, 68, 85, 102	Jacobson, 1977 Patil & Dixit, 1995

Cryptocoryne costata		34	Jacobson, 1977
Cryptocoryne crispatula		36, 54	Jacobson, 1977 Arends et al., 1982
Cryptocoryne cruddasiana		***36***	Bogner & Petersen, 2007
Cryptocoryne didericii		34	Arends et al., 1982
Cryptocoryne edithiae		34, 68	Arends et al., 1982 Petersen, 1989
Cryptocoryne elliptica		34	Petersen, 1989
Cryptocoryne ferruginea		34, 68	Arends et al., 1982 Petersen, 1989
Cryptocoryne fusca		34	Arends et al., 1982
Cryptocoryne gasseri		30, 34	Jacobson, 1977 Arends et al., 1982
Cryptocoryne grabowskii		68	Arends et al., 1982
Cryptocoryne gracilis		20	Arends et al., 1982
Cryptocoryne griffithii		34	Arends et al., 1982
Cryptocoryne hudoroi		20	Petersen, 1989
Cryptocoryne jacobsenii		34	Arends et al., 1982
Cryptocoryne keei		20, 34	Arends et al., 1982 Petersen, 1989
Cryptocoryne lingua		36	Arends et al., 1982
Cryptocoryne longicauda		30	Arends et al., 1982
Cryptocoryne longispatha		36	Marchant, 1971b
Cryptocoryne lutea		28	Jacobson, 1977
Cryptocoryne minima		34	Jacobson, 1977
Cryptocoryne moehlmannii		30	Arends et al., 1982
Cryptocoryne nevillii		28, 30	Arends et al., 1982 Petersen, 1989
Cryptocoryne nurii		34	Arends et al., 1982
Cryptocoryne pallidinervia		34	Petersen, 1989
Cryptocoryne parva		28	Jacobson, 1977
Cryptocoryne petchii		42	Jacobson, 1977
Cryptocoryne pontederiifolia		30	Jacobson, 1977
Cryptocoryne purpurea		34	Jacobson, 1977
Cryptocoryne pygmaea		34	Arends et al., 1982
Cryptocoryne retrospiralis		36, 56, 70, 72, 90	Arends et al., 1982 Jacobson, 1977 Patil & Dixit, 1995 Subramanian & Munian, 1988 Sampathkumar & Ayyangar, 1981
Cryptocoryne schulzei		34, 68	Arends et al., 1982 Petersen, 1989
Cryptocoryne scurrilis		68	Arends et al., 1982
Cryptocoryne siamensis		68	Jacobson, 1977
Cryptocoryne sp.		34	Petersen, 1993
Cryptocoryne spiralis	45	33, 66, 70, 72,	Jacobson, 1977 Ramachandran, 1978

		88, 90, 112, Ca. 132	Sarkar et al., 1976 Arends et al., 1982 Patil & Dixit, 1995 Subramanian & Munian, 1988 Petersen, 1993
Cryptocoryne striolata		20	Arends et al., 1982
Cryptocoryne thwaitesii		36, 42	Jacobsen, 1976 Marchant, 1971b
Cryptocoryne tonkinensis		36	Jacobson, 1977
Cryptocoryne tortilis		34	Arends et al., 1982
Cryptocoryne undulata		28, 42	Jacobson, 1977
Cryptocoryne usteriana		34	Jacobson, 1977
Cryptocoryne venemae		34	Arends et al., 1982
Cryptocoryne versteegii		34	Jacobson, 1977
Cryptocoryne villosa		30	Petersen, 1989
Cryptocoryne walkeri		28, 42	Arends et al., 1982 Jacobson, 1977
Cryptocoryne wendtii		28, 42	Jacobson, 1977
Cryptocoryne willisii		28	Marchant, 1971b
Cryptocoryne zonata		68	Arends et al., 1982
Cryptocoryne zukalii		34	Arends et al., 1982
Culcasia glandulosa		42	Petersen, 1989
Culcasia liberica		c. 42	Petersen, 1989
Culcasia longevaginata		42	Petersen, 1989
Culcasia orientales		42	Petersen, 1989
Culcasia ponduriformes		c. 42	Petersen, 1989
Culcasia rotundifolia		42	Petersen, 1989
Culcasia saxatilis		c. 42	Petersen, 1989
Culcasia scandes		c. 40	Petersen, 1989
Culcasia seretii		42	Petersen, 1989
Cyrtosperma chamissonis		24	Petersen, 1989
Cyrtosperma cuspidispathum		26	Petersen, 1989
Cyrtosperma ferox		26	Petersen, 1989
Cyrtosperma johnstonii		26	Petersen, 1989
Dieffenbachia amoena		34	Gireesh &. Bhavanandan, 1994
Dieffenbachia barraquiniana		34	Gireesh & Bhavanandan, 1994
Dieffenbachia baumanii		54	Petersen, 1989
Dieffenbachia bausei	c. 17	34	Petersen, 1989
Dieffenbachia eburnea		34	Damerval, 1980
Dieffenbachia exotica		34	Gireesh & Bhavanandan, 1994
Dieffenbachia hoffmannii		34	Petersen, 1989
Dieffenbachia macrophylla	17	34	Petersen, 1989
Dieffenbachia maculata		34, 40	Gireesh & Bhavanandan, 1994
Dieffenbachia memoriacorsii		34	Petersen, 1989
Dieffenbachia oerstedii		34	Petersen, 1989
Dieffenbachia picta	17	34, 36,	Ramachandran, 1978

		68	Sharma, 1970
			Subramanian & Munian, 1988
Dieffenbachia seguine		34	Petersen, 1989
Dieffenbachia splendens		34	Damerval, 1980
Dracontioides desciscens		26	Petersen, 1989
Dracontium aricuaisanum		26	Petersen, 1989
Dracontium changuango		26	Petersen, 1989
Dracontium foecundum		26	Petersen, 1989
Dracontium gigas		26	Petersen, 1989
Dracontium prancei (polyphyllum)		26	Petersen, 1989
Dracunculus canariensis		28	Petersen, 1989
Dracunculus muscivorus		56	Scrugli, 1977
Dracunculus vulgaris		28, 32	Popova & Ceschmedjiev, 1978
			Van Loon, 1982
Eminium crassipes	14	28 (56)	Petersen, 1989
Eminium koenenianum		28	Johnson & Brandham, 1997
Eminium lehmannii		28	Petersen, 1989
Epipremnum falicifolium		84	Petersen, 1989
Epipremnum mirabile		70	Sharma, 1970
Epipremnum pinnatum		60	Petersen, 1989
Filarum manserichense		28	This paper
Furtadoa sumatrensis		40	Okada, 1982
Furtadoa sumatrensis		40	Petersen, 1989
Furtadoa sumatrensis		40	Okada, 2000
Gearum brasiliense		***34,68***	Bogner & Petersen, 2007
Gonatopus (Heterolobium) petiolulatus		34	Marchant, 1971a
Gonatopus angustus		c. 68	Petersen, 1989
Gonatopus boivinii		34	Petersen, 1989
Gonatopus marattioides		34	Petersen, 1989
Gonatopus petiolulatus		34	Petersen, 1989
Gorgonidium mirabile		34	Petersen, 1989
Gorgonidium vargasii		34	Petersen, 1989
Gorgonidium vermicidum		34	Petersen, 1989
Gymnostachys anceps		48	Petersen, 1989
Hapaline benthamiana		26	Petersen, 1989
Hapaline brownii		28	Petersen, 1989
Helicodiceros muscivorus		56	Petersen, 1989
Hestia longifolia		26	This paper
Heteropsis oblongifolia		26, 28	Petersen, 1989
Holochlamys beccarii		30, 60	Petersen, 1989
			Oginuma et al., 1998
Homalomena caerulescens		40	Marchant, 1971a
Homalomena consobrina		40	Okada, 2000
Homalomena cordata		40	Petersen, 1989
Homalomena cristata		40	Petersen, 1989
Homalomena elliptica		42	Petersen, 1989
Homalomena gadutensis		38	Okada, 1985

Homalomena griffithii		40	Okada, 2000
Homalomena hastata		40	Okada, 1985
Homalomena humilis		40, 42	Petersen, 1989
Homalomena lancifolia		40	Okada, 2000
Homalomena lindenii		40, 56	Petersen, 1989
Homalomena lindenii (Alocasia lindenii)		40, 56	Sharma, 1970
Homalomena megalophylla		40	Okada, 1985
Homalomena monandra		40	Petersen, 1989
Homalomena occulta		42	Petersen, 1989
Homalomena padandensis		40	Petersen, 1989
Homalomena pendula		40	Petersen, 1989
Homalomena pygmaea		40	Okada, 1982
Homalomena rubescens		40	Petersen, 1989
Homalomena rusdii		40	Okada, 2000
Homalomena sagitifolia		40	Okada, 1982
Homalomena singaporense		40	Petersen, 1989
Homalomena speariae		42	Petersen, 1989
Homalomena sulcata		40	Okada, 2000
Homalomena wallisii		42	Petersen, 1989
Incarum pavonii		***34***	Bogner & Petersen, 2007
Jasarum steyermarkii		22	Petersen, 1989
Lagenandra bogneri		36	Petersen, 1989
Lagenandra dewitii		36	Petersen, 1989
Lagenandra erosa		36	Petersen, 1989
Lagenandra jacobsenii		36	Petersen, 1989
Lagenandra koenigii		36	Petersen, 1989
Lagenandra lancifolia		36	Marchant, 1971b
Lagenandra meeboldii		36	Petersen, 1989
Lagenandra nairii		c. 72	Petersen, 1989
Lagenandra ovata	18	32, 36	Ramachandran, 1978; Battacharya, 1975
Lagenandra praetermissa		36	Petersen, 1989
Lagenandra schulzei		36	Petersen, 1989
Lagenandra thwaitesii		36	Arends & van der Laan, 1978
Lagenandra toxicaria		36	Petersen, 1989
Lagenandra toxicaria		36	Marchant, 1971b
Landotia punctata		40	Landolt, 1986
Lasia heterophylla		26	Sharma, 1970
Lasia heterophylla (spinosa)	13	26	Ramachandran, 1978 Petersen, 1989
Lasimorpha senegalensis		26	Petersen, 1989
Lazarum (Typhonium) brownii		c. 160	Petersen, 1989
Lazarum (Typhonium) eliosurum		c. 118, 130, 152, 168	Petersen, 1989
Lazarum brownii		c. 160	Petersen, 1989

Lazarum eliosurum		c. 118, 130, 152, 168	Petersen, 1989
Lemna aequinoctialis		40	Landolt, 1986
Lemna disperma		40	Landolt, 1986
Lemna gibba		40	Landolt, 1986
Lemna japonica		40	Landolt, 1986
Lemna minor		40	Landolt, 1986
Lemna minuscula		40	Landolt, 1986
Lemna obscura		40	Landolt, 1986
Lemna perpusilla		40	Landolt, 1986
Lemna trisulca		40	Landolt, 1986
Lemna turionifera		40	Landolt, 1986
Lemna valdiviana		40	Landolt, 1986
Lysichiton americanus		28	Petersen, 1989
Lysichiton camtschatcensis		28	Sokolovskaya & Probatova, 1985
Mangonia tweediana		***34***	Bogner & Petersen, 2007
Monstera acuminata		60	Petersen, 1989
Monstera adansonii		60	Petersen, 1989
Monstera deliciosa		24, 56, 58, 60	Chaudhuri & Sharma, 1979 Huang et al., 1989
Monstera friedrichsthalii		60	Marchant, 1970
Monstera spruceana (Alloschemone occidentalis)		60	Petersen, 1989
Montrichardia arborescens		48	Petersen, 1989
Nephthytis afzelli		60	Marchant, 1971a
Nephthytis bintuluensis		36	Hay, A., J. Bogner & P. C. Boyce 1994
Nephthytis hallaei		40	Petersen, 1989
Nephthytis poissonii		60	Marchant, 1971a
Nephthytis swainei		40	Petersen, 1989
Ooia (Piptospatha) grabowskii		26	Petersen, 1989
Orontium aquaticum	13	26	Petersen, 1989 Petersen, 1989
Pedicellarum paiei		***24***	Bogner & Petersen, 2007
Peltandra virginica		112	Marchant, 1971a
Philodendron andreanum		32, 34	Sharma, 1970 Petersen, 1989
Philodendron bipinnatifidum	18	36	Petersen, 1989
Philodendron cannifolium		34	Petersen, 1989
Philodendron cordatum		34	Petersen, 1989
Philodendron cuspidatum		30 (32), 36	Chaudhuri & Sharma, 1979 Petersen, 1989
Philodendron erubescens		32	Petersen, 1989
Philodendron eximium		34	Petersen, 1989

Philodendron giganteum		30, 34	Petersen, 1989
Philodendron glandifolium		34	Petersen, 1989
Philodendron gloriosum		34	Petersen, 1989
Philodendron hastatum		34	Petersen, 1989
Philodendron houlletianum		32	Petersen, 1989
Philodendron imbe	17	34	Petersen, 1989
Philodendron lacerum		36	Petersen, 1989
Philodendron laciniosum		32	Petersen, 1989
Philodendron lundii		36	Petersen, 1989
Philodendron melinonii		30	Petersen, 1989
Philodendron micans		32	Petersen, 1989
Philodendron panduraeforme		34	Petersen, 1989
Philodendron pittieri		34	Petersen, 1989
Philodendron radiatum		32 + B	Petersen, 1989
Philodendron rugosum		36	Petersen, 1989
Philodendron scandens		30, 32	Petersen, 1989 Subramanian & Munian, 1988
Philodendron selloum		32, 34, 36, 48	Subramanian & Munian, 1988 Chaudhuri & Sharma, 1979 Petersen, 1989
Philodendron sodiroi		34	Petersen, 1989
Philodendron speciosum		36	Petersen, 1989
Philodendron sqaumiferum		26, 34	Petersen, 1989
Philodendron undulatum	18	36	Petersen, 1989
Philodendron verrucosum	17	34	Petersen, 1989
Philodendron warscewiczii		34	Petersen, 1989
Philodendron wendlandii		54	Subramanian & Munian, 1988
Philonotion americanum		26	This paper
Phymatarum borneense		26, 28	Petersen, 198
Pichinia disticha		26	This paper
Pinellia cordata		26, 72, 78	Li et al., 1997 Yi et al., 2005
Pinellia integrifolia		78	Yi et al., 2005
Pinellia major		20	Petersen, 1989
Pinellia pedatisecta		26	Li et al., 1997
Pinellia peltata		78	Li et al., 1997
Pinellia polyphylla		26	Yi et al., 2005
Pinellia ternata		28, 42, 54, 72, 78 90, 91, 99, 104, 108, 115, 116,	Li et al., 1997; Cheng et al.; 1991 Gu & Hsu, 1991 Wang & Peng, 2000 Marchant, 1971b

		117, 128	
Pinellia tripartita	26	26, 52	Petersen, 1989
Pinellia yaoluopingensis		26	Li et al., 1997
Piptospatha burbidgei		26 + Bs	Okada, 2000
Piptospatha elongata		26, 39	Okada, 2000
Piptospatha insignis		26	Petersen, 1989
Piptospatha perakensis		26	Petersen, 1989
Piptospatha ridleyi		26	Petersen, 1989
Piptospatha ridleyi		26	Petersen, 1989
Piptospatha truncatum		26	Okada, 2000
Pistia stratiotes	12	14, 28	Subramanian & Munian, 1988 Petersen, 1989
Podolasia stipitata		26	Petersen, 1993
Pothoidium lobbianum		24	Petersen, 1989
Pothos chapelieri		24	Marchant, 1973
Pothos scandens	12	24, 36	Sarkar, 1991 Petersen, 1989
Pothos viridis		60	Sharma, 1970
Protarum sechellarum		28	Petersen, 1989
Pseudodracontium lacourii		26	Petersen, 1989
Pseudodracontium siamense		26	Petersen, 1989
Pseudohydrosme gabunensis		Ca. 40	Petersen, 1989
Pycnospatha arietina		26	Petersen, 1989
Pycnospatha arietina (soerensenii)		26	Marchant, 1973
Pycnospatha palmata		***26***	Bogner & Petersen, 2007
Remusatia hookeriana		28	Gu et al., 1992
Remusatia ornata		42	Long et al., 1989
Remusatia ornatus		30	Kuruvilla et al., 1989
Remusatia pumila		20	Li & Hay, 1992
Remusatia pumila (sarmentosus)		28	Sharma, 1970
Remusatia pumilus		28	Petersen, 1989
Remusatia vivipara		28, 42, 56	Li & Hay, 1992 Marchant, 1971a
Rhaphidophora beccarii		60	Okada, 2000
Rhaphidophora bogneri		60	Petersen, 1989
Rhaphidophora celatocaulis		56, 60	Petersen, 1989 Marchant, 1970
Rhaphidophora decursiva		26, 54, 56	Chaudhuri & Sharma, 1979 Sarkar et al., 1976 Petersen, 1989
Rhaphidophora glauca		56	Chaudhuri & Sharma, 1979
Rhaphidophora lancifolia		56	Sharma, 1970
Rhaphidophora peepla		42, c. 120	Sharma, 1970 Petersen, 1989

Rhaphidophora pteropoda	60	Petersen, 1989
Rhodospatha blanda	56 + f	Petersen, 1989
Rhodospatha hastata	60	Petersen, 1989
Rhodospatha picta	28	Petersen, 1989
Sauromatum (Typhonium) diversifolium	52	Mehra & Sachdeva, 1976
Sauromatum (Typhonium) giganteum	52	Petersen, 1989
Sauromatum gaoligongense	26	Cusimano et al., 2010
Sauromatum giganteum (Typhonium giraldii)	54	Petersen, 1989
Sauromatum guttatum	26	Chaudhuri & Sharma, 1979
Sauromatum hirsutum	26	Cusimano et al., 2010
Sauromatum horsfieldii (Typhonium larsenii)	26	Petersen, 1989
Sauromatum tentaculatum	26	Cusimano et al., 2010
Sauromatum venosum	26, 52, 104	Sarkar, A. K. 1991
Scaphispatha gracilis	28	Petersen, 1989
Schismatoglottis bulbifera	26	Okada, 2000
Schismatoglottis calyptrata	26	Okada, 1982
Schismatoglottis celebica	26	Okada, 2000
Schismatoglottis erecta	26	Okada, 2000
Schismatoglottis hayana	***26***	Bogner & Petersen, 2007
Schismatoglottis homalomenoidea	26	Okada, 2000
Schismatoglottis irrorata	52 + Bs	Okada, 2000
Schismatoglottis lancifolia	26 + Bs, 39 + Bs	Okada, 2000
Schismatoglottis multiflora	26	Okada, 2000
Schismatoglottis parvifolia	26	Okada, 2000
Schismatoglottis picta	30, 52	Sharma, 1970 Petersen, 1989
Schismatoglottis roseospatha	26	Petersen, 1989
Schismatoglottis tecturata	52	Petersen, 1989
Schismatoglottis triandra	26	Petersen, 1989
Schismatoglottis wallichii	26	Petersen, 1989
Schottariella mirifica	--	Not counted
Scindapsus aureus	48	Subramanian & Munian, 1988 Sharma, 1970
Scindapsus hederaceus	64	Okada, 1982
Scindapsus latifolius	58	Petersen, 1989
Scindapsus lucens	60	Petersen, 1993
Scindapsus megaphyllus	56	Huang et al., 1989

Scindapsus officinalis		56	Chaudhuri & Sharma, 1979
Scindapsus perakensis		60	Petersen, 1989
Scindapsus pictus		60, 70, 112	Sharma, 1970 Petersen, 1989
Spathantheum intermedium		34	Bogner, 1997
Spathantheum orbignyanum		34	Petersen, 1993
Spathicarpa sagittifolia	17	34	Petersen, 1989
Spathiphyllum cannaefolium	15	30	Jos & Rajendran, 1976 Petersen, 1989
Spathiphyllum cochlearispathum		30	Damerval, 1980
Spathiphyllum commutatum		c. 30	Petersen, 1989
Spathiphyllum floribundum	30	30, 60	Petersen, 1989
Spathiphyllum friedrichsthalii		30	Marchant, 1973
Spathiphyllum grandifolium		30	Petersen, 1989
Spathiphyllum harveyanum	15	30	Petersen, 1989
Spathiphyllum patinii	9	18, 30	Petersen, 1989
Spathiphyllum wallisii		30	Petersen, 1989
Spirodela intermedia		30	Landolt, 1986
Spirodela polyrriza		30	Landolt, 1986
Stenospermation popayanense		28	Petersen, 1989
Stenospermation popayense		28	Marchant, 1970
Stenospermation robustum		28	Petersen, 1989
Stenospermation sodiroanum		28	Petersen, 1989
Steudnera colocasiifolia		36	Sharma, 1970
Steudnera colocasioides		28	Kuruvilla et al., 1989
Steudnera discolor	16	56	Jos et al., 1971 Petersen, 1989
Steudnera henryana		28	Petersen, 1993
Stylochaeton bogneri		56	Petersen, 1989
Stylochaeton puberulus		28	Petersen, 1989
Stylochaeton salaamicus		28	Petersen, 1989
Stylochaeton zenkeri		56	Petersen, 1989
Symplocarpus foetidus		60	Blair, A. 1975
Symplocarpus renifolius		30, 60	Sokolovskaya & Probatova, 1985 Petersen, 1989
Synandrospadix vermitoxicus	17	34	Petersen, 1989
Syngonium albolineatum		22	Subramanian & Munian, 1988
Syngonium auritum		24	Guha & Bhattacharya, 1987

Syngonium erythrophyllum		28	Petersen, 1989
Syngonium hastifolium		28	Sharma, 1970
Syngonium macrophyllum		24	Guha & Bhattacharya, 1987
Syngonium podophyllum	12	24, 26	Guha & Bhattacharya, 1987; Petersen, 1989
Syngonium steyermarkii		28	Petersen, 1989
Syngonium vellozianum		26	Marchant, 1971b
Syngonium wendlandii		24	Guha & Bhattacharya, 1987
Taccarum weddellianum		34	Petersen, 1989
Theriophonum dalzellii	8	16	Jayalakshmi, 1994 Petersen, 1989
Theriophonum indicum	8	16	Ramachandran, 1978
Theriophonum infaustum		16	Ramachandran, 1978
Theriophonum minutum	8	14, 16, 24	Ramachandran, 1978 Subramanian & Munian, 1988 Jayalakshmi, 1994
Theriophonum sivaganganum		32	Jayalakshmi, 1994
Typhonium baoshanense		10	Zhin-Lin et al., 2007
Typhonium blumei		52	Wang &. Yang, 1996
Typhonium bulbiferum	10	20	Ramachandran, 1978 Petersen, 1989
Typhonium flagelliforme	8	16	Petersen, 1989
Typhonium flagelliforme (cuspidatum)	8	16	Ramachandran, 1978
Typhonium inopinatum	13	26	Petersen, 1989
Typhonium jinpingense		10	Zhoglang et al., 2002
Typhonium roxburghii		(26), 52	Petersen, 1989
Typhonium roxburghii (divaricatum)	26	16, 52, 65	Ramachandran, 1978 Jos et al., 1971
Typhonium trilobatum		18, 26, 36	Ramachandran, 1978 Chaudhuri & Sharma, 1979
Typhonodorum lindleyanum		112	Petersen, 1989
Ulearum donburnsii		***14***	Bogner & Petersen, 2007
Ulearum viridispadix		14	Petersen, 1989
Urospatha sagittifolia		52	Petersen, 1989
Wolffia angusta		40	Landolt, 1986
Wolffia arrhiza		40	Landolt, 1986
Wolffia australiana		20, 40	Landolt, 1986
Wolffia borealis		40	Landolt, 1986
Wolffia brasiliensis		40	Landolt, 1986
Wolffia columbiana		40	Landolt, 1986
Wolffia globosa		40	Landolt, 1986
Wolffia microscopica		40	Landolt, 1986
Wolffiela denticulata		20, 40	Landolt, 1986
Wolffiela gladiata		40	Landolt, 1986
Wolffiela hyalina		40	Landolt, 1986
Wolffiela lingulata		20, 40	Landolt, 1986
Wolffiela neotropica		40	Landolt, 1986

Wolffiela oblonga		40	Landolt, 1986
Wolffiela welwitschii		40	Landolt, 1986
Xanthosoma alrovirens		26	Marchant, 1971a
Xanthosoma brasiliense		26	Petersen, 1989
Xanthosoma helleborifolium		39	Petersen, 1989
Xanthosoma mariae		***26***	Bogner & Petersen, 2007
Xanthosoma nigrum		c. 26	Petersen, 1989
Xanthosoma pentaphyllum		26	Petersen, 1989
Xanthosoma plowmanii		26	Petersen, 1989
Xanthosoma robustum	13		Petersen, 1989
Xanthosoma sagittifolium		26	Udengwu & Okafor, 1999
Xanthosoma striatipes		22	Petersen, 1993
Xanthosoma violaceum		26	Udengwu & Okafor, 1999
Zamioculcas zamiifolia	17	34	Petersen, 1989
Zantedeschia aethiopica	16	32	Yao et al., 1994 Petersen, 1989
Zantedeschia albo-maculata	16	32	Petersen, 1989
Zantedeschia elliottiana	16	32	Yao et al., 1994; Petersen, 1989
Zantedeschia odorata		32	Yao et al., 1994
Zantedeschia pentlandii		32	Yao et al., 1994
Zantedeschia rehmannii	16	32	Yao et al., 1994; Petersen, 1989
Zantedeschia tropicalis		32	Marchant, 1971a
Zomicarpa pythonium		22	Petersen, 1989
Zomicarpa riedelianum		20	Petersen, 1989
Zomicarpella amazonica		26	Bogner, 1997

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Chapter 3

Combining FISH and model-based predictions to understand chromosome evolution in *Typhonium* (Araceae)

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Combining FISH and model-based predictions to understand chromosome evolution in *Typhonium* (Araceae)

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• *Background and Aims* Since the advent of molecular phylogenetics, numerous attempts have been made to infer the evolutionary trajectories of chromosome numbers on DNA phylogenies. Ideally, such inferences should be evaluated against cytogenetic data. Towards this goal, we carried out phylogenetic modelling of chromosome number change and fluorescence *in situ* hybridization (FISH) in a medium sized genus of Araceae to elucidate if data from chromosomal markers would support maximum likelihood-inferred changes in chromosome numbers among close relatives. *Typhonium*, the focal genus, includes species with $2n = 65$ and $2n = 8$, the lowest known count in the family.
• *Methods* A phylogeny from nuclear and plastid sequences (96 taxa, 4252 nucleotides) and counts for all included species (15 of them first reported here) were used to model chromosome number evolution, assuming discrete events, such as polyploidization and descending or ascending dysploidy, occurring at different rates. FISH with three probes (5S rDNA, 45S rDNA and *Arabidopsis*-like telomeres) was performed on ten species with $2n = 8$ to $2n = 24$.
• *Key Results* The best-fitting models assume numerous past chromosome number reductions. Of the species analysed with FISH, the two with the lowest chromosome numbers contained interstitial telomeric signals (*Its*), which together with the phylogeny and modelling indicates decreasing dysploidy as an explanation for the low numbers. A model-inferred polyploidization in another species is matched by an increase in rDNA sites.
• *Conclusions* The combination of a densely sampled phylogeny, ancestral state modelling and FISH revealed that the species with $n = 4$ is highly derived, with the FISH data pointing to a Robertsonian fusion-like chromosome rearrangement in the ancestor of this species.

Key words: Ancestral trait reconstruction, 5S rDNA, 45S rDNA, telomeres, FISH, Bayesian inference, maximum likelihood inference, aneuploid chromosome numbers, chromosome evolution, *Typhonium*, Araceae.

INTRODUCTION

Araceae are a large family of monocotyledons (3300 species, 117 genera; Boyce and Croat, 2013) that are phylogenetically well understood (Cusimano *et al.*, 2011; Nauheimer *et al.*, 2012). Many of the species are in cultivation, and chromosome counts are available for 862 species (26 % of the family), ranging from $2n = 10$ to $2n = 168$ (Cusimano *et al.*, 2012*a*: Supplementary Data Table S1 provides species names and original references). The family's range of chromosome numbers, phylogenetic framework and often easy cultivation (i.e. access to root tips) make Araceae suitable for bringing together modern methods of cytogenetics and ancestral trait reconstruction to advance our understanding of genome evolution and organization. As a first step, we recently inferred chromosome evolution using a genus-level phylogeny and maximum likelihood models of chromosome number change (Cusimano *et al.*, 2012*a*; model details are given in the Materials and Methods). The results suggested an ancestral haploid number of 16 or 18, rather than the base numbers of $x = 7$ (Larsen, 1969; Marchant, 1973) or $x = 14$ (Peterssen, 1993) previously hypothesized without consideration of phylogenetic relationships and probably overweighting derived chromosome numbers because the early-branching groups, such as Lemnoideae, which have relatively high chromosome numbers, were not yet included in Araceae. Other results were a limited role for polyploidization and numerous reductions of chromosome numbers.

Typhonium, a Southeast Asian clade of 50–60 species, has already been the focus of studies in our lab treating its circumscription, natural geographic range and diversification rate (Cusimano *et al.*, 2010, 2012*b*). Prior to the present study, chromosome counts were available for only 10 of its species and ranged from $2n = 10$ (in *Typhonium baoshanense* and *T. jinpingense*; Zhonglang *et al.*, 2002; Zhin-Lin *et al.*, 2007) to $2n = 65$ [*Typhonium roxburghii*, Cusimano *et al.*, 2012*a*; Index to Plant Chromosome Numbers (IPCN) www.tropicos.org/Project/IPCN]. For the present study, we added new chromosome counts for another 15 species of the genus. *Typhonium* is embedded among genera with chromosome numbers based on $n = 13$ or 14 (*Arisaema*, *Pinellia*, *Sauromatum*, *Biarum*, *Helicodiceros*, *Dracunculus* and *Arum*); only *Theriophonum* has $n = 8$. In our family-wide study, which included just one species per genus, we inferred a reduction from an ancestral number $a = 14–13$ in this group (Cusimano *et al.*, 2012*a*). We here enlarge the phylogeny for *Typhonium* and its relatives and use the new and published chromosome counts to understand the chromosomal changes at a finer scale.

To achieve this, we selected ten species for fluorescence *in situ* hybridization (FISH) experiments, chosen to represent the range from $2n = 24$ to $2n = 8$, the lowest number in the family (newly

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reported in this study). By applying three probes, 5S rDNA, 45S rDNA and an *Arabidopsis*-type telomeric probe – and with the more densely sampled phylogeny with more counted species – we hoped to test the previously inferred direction from higher to lower numbers via chromosome 'loss' and to be able to infer mechanisms behind numerical changes. Mechanisms detectable with FISH are structural changes associated with primary chromosome rearrangements (insertions, deletions, duplications, reciprocal translocations and sequence amplification) or secondary chromosome rearrangements (replication slipping) (Schubert, 2007). Recent examples of such inferences based on FISH come from *Hypochaeris* and *Nothoscordum arenarium* (Weiss-Schneeweiss *et al.*, 2008; Souza *et al.*, 2009). FISH can also help detect recent polyploidization, i.e. duplication of an organism's entire set of chromosomes, or dysploidy, i.e. an increase or decrease in chromosome number related to chromosome rearrangements, especially when used in a phylogenetic framework. For instance, the number of 5S rDNA and 45S rDNA sites sometimes doubles with polyploidization (Ansari *et al.*, 2008; Weiss-Schneeweiss *et al.*, 2008; Souza *et al.*, 2010). Similarly, decreasing dysploidy inferred from a phylogeny would be supported by the discovery of interstitial telomeric signals. Such signals are sometimes found following fusion–fission cycles, and with probes homologous to plant telomeric repeats they can be visualized (Schubert, 1992; Fuchs *et al*., 1995). Since several mechanisms can lead to interstitial telomere signals, a careful consideration of the specific karyotype(s) being analysed is always required, but in principle the distribution of telomeric signals can suggest chromosome loss by fusion.

MATERIALS AND METHODS

Sampling of taxa and molecular markers

We sampled the 96 species and subspecies of Areae tribe plus outgroups listed in Supplementary Data Table S1, which also provides information on vouchers, DNA loci sequenced and GenBank accession numbers. Seventy-nine sequences were newly generated for this study. The taxon sample covers all but one genus of the Areae [*Arum*, *Biarum*, *Dracunculus*, *Helicodiceros*, Australian *Typhonium* (= *Lazarum*), *Sauromatum*, *Theriophonum* and *Typhonium*]. Only *Eminium* is not included due to lack of chromosome counts. As outgroups, we used a species of *Alocasia*, 24 of *Arisaema* (one with two accessions) and five of *Pinellia*. Only species with known chromosome numbers are included. *Typhonium* itself is represented by 22 of its 50–60 species (one species is represented by two accessions).

To infer phylogenetic relationships, we relied on part of the nuclear phytochrome C gene (*PhyC*) and two chloroplast loci, the *rpl20–rps12* intergenic spacer and part of the lysine tRNA gene (*trnK*), which contains the maturase K intron (*matK*). Total DNA from silica-dried leaves was extracted with the NucleoSpin Plant II kit according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany). Amplification and sequencing were performed using the primers described in Cusimano *et al.* (2010). Polymerase chain reactions were performed using 1·25 U of *Taq* DNA polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany) and the following cycle conditions: the initial step of 3 min at 94 °C was followed by 39 cycles of 94 °C for 30 s for DNA denaturation, 54 °C for 60 s for primer annealing, 68 °C for 90 s for primer extension and 68 °C for 10 min after the final cycle. The PCR products were purified with Exo I and FastAP (Fermentas, St Leon-Rot, Germany). Sequencing was done on an ABI 3130-4 capillary sequencer, and sequences were assembled and edited with Sequencher 4·2 (Gene Codes Cooperation, Ann Arbor, MI, USA). All contigs were BLAST-searched in GenBank, which for nuclear sequences provides a check against fungal contamination and for plastid sequences a check against DNA from leaf epiphytes.

Phylogenetic analyses

Alignments were generated in MAFFT (http://mafft.cbrc.jp/alignment/server/) and checked visually using MEGA5 (Tamura *et al.*, 2011). To remove poorly aligned positions, single alignments were exported to a server running Gblocks vs. 0·91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with the less stringent options selected (Castresana, 2000). The plastid and nuclear data were first analysed separately and, in the absence of statistically supported topological contradictions (>80 %), they were combined. The combined matrix (4252 aligned nucleotides) was used for maximum likelihood (ML) tree searches in RAxML (Stamatakis, 2006; Stamatakis *et al.*, 2008), using the GTR + G substitution model with four rate categories. Bootstrapping under ML used 1000 replicates. We also generated ultrametric trees in BEAST v. 1.7.5 (Drummond and Rambaut, 2007), using the same substitution model and a pure-birth Yule model as the tree prior. The analysis was run for 10 million generations, sampling every 1000th step. The burn-in fraction, i.e. the number of trees to be discarded from the consensus tree (the maximum clade credibility tree), was assessed using Tracer v. 1.4.1, which is part of the BEAST package.

Inference of chromosome number change

To infer ancestral haploid chromosome numbers, we relied on ChromEvol v. 1.3 of Mayrose *et al.* (2010). This lets users chose among eight models of chromosome number change that have the following six parameters: polyploidization (chromosome number duplication) with rate ρ, demi-polyploidization (polyploids derived from the fusion of gametes with different ploidy levels) with rate μ, and dysploidization (ascending, chromosome gain rate λ; descending, chromosome loss rate δ) as well as two linear rate parameters, λ_1 and δ_1, for the dysploidization rates λ and δ, allowing them to depend on the current number of chromosomes. Four of the models have a constant rate, whereas the other four include the two linear rate parameters. Both model sets also have a null model that assumes no polyploidization events. We fitted all models to the data, using either an ML phylogram or an ultrametric BEAST maximum clade probability tree, in each case with 10 000 simulated repetitions to compute the expected number of changes of the four transition types along each branch of the phylogeny. The maximum number of chromosomes was set to 10-fold higher than the highest number found in the empirical data, and the minimum number was set to 1. The root node was fixed to $a = 14$, based on our previous family-wide analysis (Cusimano *et al.*, 2012*a*).

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Model fit was assessed using the Akaike information criterion (AIC). Mayrose *et al.* (2010) have shown that accurate reconstructions of ancestral chromosome numbers and events are only obtained from trees with intermediate evolutionary distances. We therefore adjusted the phylogram and ultrametric tree such that both had a total length of 0·2, which could be achieved by multiplying all branch lengths by suitable factors. Results were plotted in R using the ChromEvol functions version 0.9-1 of N. Cusimano (http://www.sysbot.biologie.uni-muenchen.de/en/people/cusimano/use_r.html).

Chromosome preparation, FISH analyses, DNA probes and C-banding

Bulbs of *Typhonium* were cultivated in the greenhouses of the Munich Botanical Garden, and, for most, plenty of root tips were available although usually only from a single individual. They originally came from W. Hetterscheid's taxonomic studies on *Typhonium* (Hetterscheid and Boyce, 2000; Hetterscheid and Nguyen, 2001; Hetterscheid *et al.*, 2001; Hetterscheid and Galloway, 2006; Hetterscheid, 2013). The chromosomes of 15 species (single individuals) were newly counted, namely *T. circinnatum*, *T. corrugatum*, *T. echinulatum*, *T. filiforme*, *T. gallowayi*, *T. huense, T. laoticum*, *T.* spec. H.AR. 664 (morphologically similar to *T. laoticum*, but clearly a separate species based on the molecular results), *T. orbifolium*, *T. saraburiense*, *T. stigmatilobatum*, *T. tubispathum*, *T. violifolium*, *Typhonium* spec. 17 Thailand, and *T. trilobatum*. Authors of species names and voucher material for each species are given in Supplementary Data Table S1.

Root tips were pre-treated in 2 mM 8-hydroxyquinoline for 20 h at 4 °C, fixed in freshly prepared 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and kept at −20 °C. For chromosome preparations, fixed root tips were washed three times for 5 min in distilled water, digested with 1 % cellulase (w/v; Onozuka RS, Serva), 0·4 % pectolyase (w/v; Sigma), 0·4 % cytohelicase (w/v; Sigma) in citric buffer, pH 4·8 for 30 min at 37 °C, dissected in a drop of 45 % acetic acid and squashed. Coverslips were removed after freezing in dry ice, and preparations were air-dried at room temperature. The quality of spreads was checked microscopically using phase contrast, and only preparations with at least ten well-spread metaphases were used for FISH. For *T. filiforme*, *T. gallowayi*, *T. orbifolium*, *T. tubispathum* and *Typhonium* spec. 17 Thailand, only a few cells per species (1–5) were counted. Pictures were taken using 4′,6-diamidino-2-phenylindole (DAPI) staining (*T.* spec. 17 Thailand) and without staining using a phase contrast microscope.

We performed FISH with a telomeric probe, and 5S rDNA and 45S rDNA probes; the telomeric probe was not used on *T. violifolium* because of a shortage of suitable material. To locate rDNAs, we used the 18S–5·8S–25S rDNA repeat unit of *Arabidopsis thaliana* in the pBSK+ plasmid, labelled with digoxigenin-11-dUTP (Roche) by nick translation, and a 349 bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris* cloned into pBSK+ (Schmidt *et al.*, 1994), labelled with biotin-16-dUTP (Roche) by PCR. The *Arabidopsis*-like telomeric probe was amplified by PCR according to Ijdo *et al.* (1991) using the oligomer primers (5′-TTTAGGG-3′)$_5$ and (5′-CCCTAAA-3′)$_5$, and labelled with digoxigenin-11-dUTP

TABLE 1. *Inferred chromosome number evolution in the Areae and their immediate outgroups under the best-fitting model, the linear-rates model with the duplication (polyploidzation) rate different from the demi-duplication rate*

Tree	Factor	Total tree length	Root tip length	Best model	LogLik	AIC	Rates				Number of events				
							λ	δ	ρ	μ	Gains	Losses	Duplications	Demi.	Total events
Ultrametric	4·5	3·5	0·045	lrde	−262·3	536·5	0·33	15·21	10·39	2·23	6·5	31·1	33·4	5	76
Phylogram	5	2·1	0·04	crde	−329·2	666·4	1·78	22·9	17·26	4·26	2·5	38·3	31·2	8·9	80·9

Column two refers to the factor used to multiply branch lengths to obtain a suitable root to tip length for the tree (see the Materials and Methods); columns three and four give the lengths obtained after adjusting branch lengths by the multiplication factor; column six gives the logarithmic likelihood; and column seven the AIC scores to the likelihood ratio tests.

The symbols for the rates inferred for all events in the tree are λ, chromosome gain rate; δ, chromosome loss rate; ρ, duplication rate; μ, demi-duplication rate.

The number of events refers to the four event types with an expectation >0·5 (demi., demi-duplication).

The last column shows the total number of events inferred on the respective tree.

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by nick translation. Hybridization mixes consisted of 50 % formamide (w/v), 2× SSC, 10 % dextran sulfate (w/v) and 70–200 ng of labelled probe. The hybridization mix was denatured at 75 °C for 10 min and immediately cooled on ice for 10 min; 10–15 μL of the mix was then added to each slide. Hybridization was carried out in a humid chamber at 37 °C for 20 h. The 5S rDNA was detected with streptavidin–Cy3 conjugate (Sigma), and the 45S rDNA with anti-digoxigenin–fluorescein isothiocyanate (FITC) conjugate (Roche) at 37 °C for 1 h. The chromosomes were counterstained with DAPI (2 μg mL^{-1}) and mounted in Vectashield (Vector). Slides first analysed with telomeric and 5S rDNA probes were de-stained, and a second hybridization was performed with 45S rDNA to obtain a sequential staining with both markers in a single cell. For more details, see Sousa *et al.* (2013).

To study a supernumerary chromosome discovered in *T. trilobatum*, we performed C-banding and FISH using the nuclear ribosomal internal transcribed spacer 2 (ITS2) of this species. The ITS2 of *T. trilobatum* was amplified by PCR using primers ITS3 and ITS4 (White *et al.*, 1990). The resulting DNA fragment (KC478077) was cloned into the pGEM-T Easy plasmid (Promega, Mannheim, Germany), sequenced and PCR-labelled with biotin-16-dUTP (Roche). Procedures for chromosome preparation, post-hybridization washes and C-banding follow Sousa *et al.* (2013).

Images were taken with a Leica DMR microscope equipped with a KAPPA-CCD camera and the KAPPA software. They were optimized for optimum contrast and brightness using Adobe Photoshop CS3 version 10.0.

RESULTS

New chromosome counts for 15 Typhonium *species*

The new chromosome counts for 15 *Typhonium* species range from $2n = 8$, the lowest number reported so far for the Araceae family, to $2n = 24$ (Table 2). Of the 15 species, five displayed odd chromosome numbers. Prior to our study, an aneuploid number, namely $2n = 65$, had only been reported for *T. roxburghii* (as *T. divaricatum*) (Ramachandran, 1978), but in other genera, such as *Amorphophallus*, *Anthurium*, *Apoballis*, *Arisaema*, *Arum*, *Caladium*, *Pinellia* and *Schismatoglottis*, aneuploidy is well documented (Cusimano *et al.*, 2012*a*). For *Anthurium* and *Schismatoglottis*, the aneuploid numbers have been discussed as possible B chromosomes (Cusimano *et al.*, 2012*a*).

Ancestral state reconstructions for Typhonium *chromosome numbers*

The combined matrix of nuclear and chloroplast markers (96 species and subspecies, 22 of them *Typhonium*; 4252 nucleotides) yielded a well-supported phylogeny (Fig. 1). We then used either the ML phylogram or an ultrametric tree (see the Materials and Methods), and chromosome counts for all 96 accessions, to model chromosome evolution, fitting all models implemented in the ChromEvol program and comparing their likelihood using AIC scores. A reconstruction on an ultrametric tree is shown in Fig. 1 and a reconstruction on a phylogram is shown in Supplementary Data Fig. S1. The statistical support for both is shown in Supplementary Data Figs S2 and S3. The best-fitting models differ slightly, depending on the tree's overall branch lengths, which is their only difference, and is shorter in the phylogram than in the ultrametric tree (phylogram 2·1 vs. ultrametric tree 3·5; Table 1). On the phylogram, the four-parameter-constant-rate model, which assumes constant gain and loss rates and a polyploidization rate that differs from the demi-polyploidization rate, best explained the empirical numbers at the tips of the tree (AIC = 666·2). On the ultrametric tree, the six-parameter-linear-rate model, which includes additional parameters for the gain and loss rates (making them linearly dependent on the current chromosome number), best explained the empirical data (AIC = 536·5). The inferred rates of change and numbers of events on the two trees are summarized in Table 1.

TABLE 2. Typhonium *species investigated with their chromosome number, presence of interstitial telomeric signals* (Its) *and distribution of 5S and 45S rDNA sites*

Species	2*n*	*Its*	No.	5S rDNA	No.	45S rDNA
Typhonium circinnatum	24	–	1	Sub-terminal	8	Interstitial/terminal
T. violifolium	22	–	1	Sub-terminal	2	Terminal
T. corrugatum	20	–	1	Interstitial	2	Terminal
T. trilobatum	19	–	1	Sub-terminal	2	Terminal
T. saraburiense	18	–	1	Sub-terminal	2	Terminal
T. echinulatum	18	–	1	Sub-terminal	2	Terminal
T. huense	15	–	1	Interstitial	**2**	Terminal
T. stigmatilobatum	15	–	1	Interstitial	**2**	Terminal
T. laoticum	9	2	1	Proximal	1	Terminal
T. spec. H.AR. 664	8	5	1	Interstitial	2	Terminal
*T. filiforme**	12	–	–	–	–	–
*T. gallowayi**	20	–	–	–	–	–
*T. orbifolium**	12	–	–	–	–	–
T. spec. 17 Thailand*	19	–	–	–	–	–
*T. tubispathum**	10	–	–	–	–	–

Authors of species names and voucher information are given in Supplementary Data Table S1.
An asterisk marks species for which only chromosome counts were obtained.
Atypical numbers of 45S rDNA sites (five instead of four) are shown in bold.

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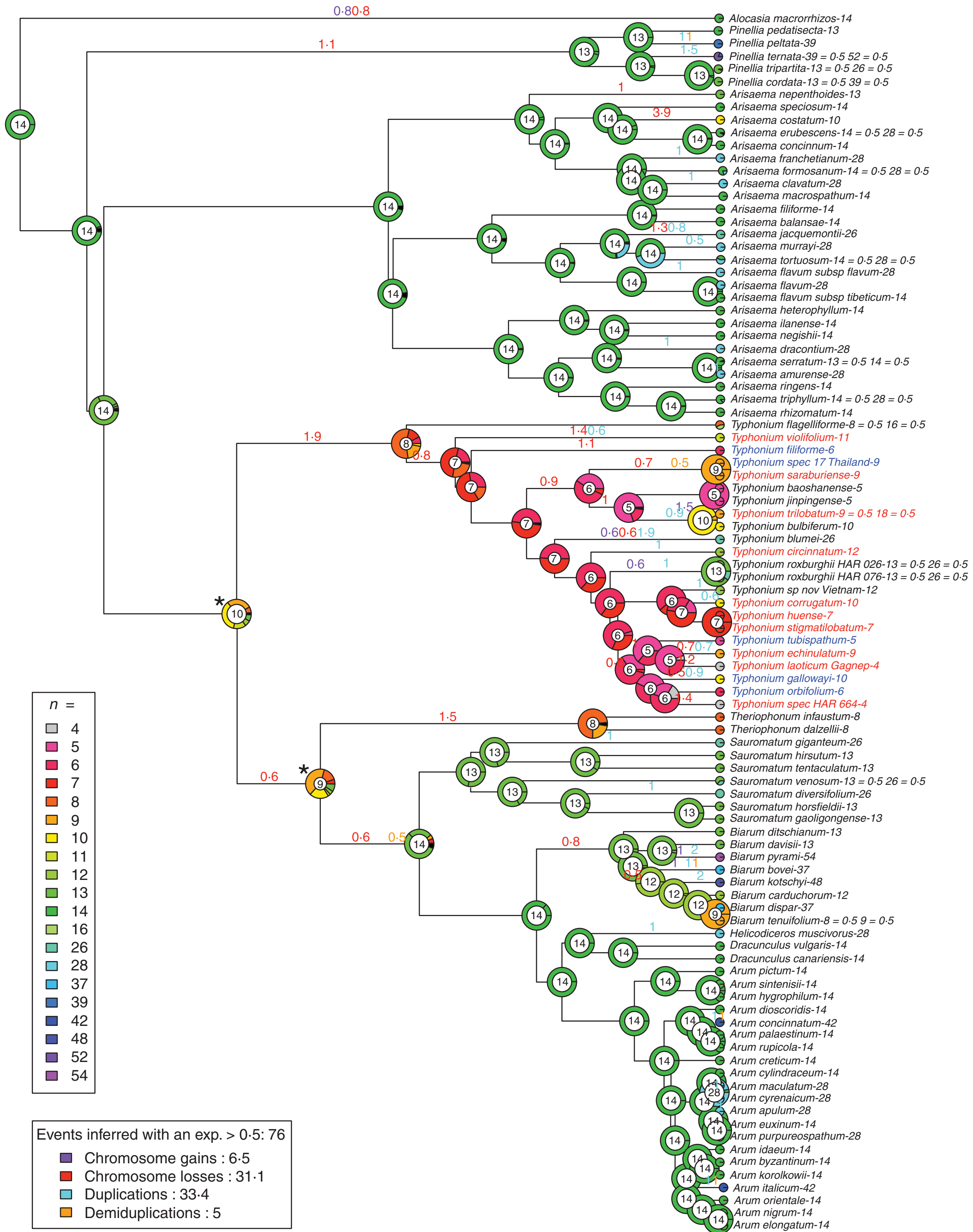

FIG. 1. Chromosome number reconstruction for the Areae on an ultrametric tree, rooted on *Alocasia macrorrhizos*. Pie charts represent the probabilities of inferred numbers, with the number inside a pie having the highest probability. Numbers above branches are colour coded by event type (gains, losses, duplications and demi-duplication) as shown in the rectangular inset, and represent the frequency with which an event type(s) with a probability >0·5 occurred along a branch. The colour coding of chromosome numbers is explained in the elongate inset on the left. Problematic inferences on the backbone are marked with an asterisk. Species investigated by FISH are labelled in red; species with only chromosome counts are labelled in blue.

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The inferred chromosome gains, losses, duplication (polyploidization) and demi-duplications are shown in the insets in the lower left of Fig. 1, and Supplementary Data Figs S1 and S3. The number of events with an expectation >0·5 is similar on the phylogram and the ultrametric tree (80·6 vs. 76; Table 1). The predominant events were chromosome losses and duplications (31·1 vs. 33·4 on the ultrametric tree), with the number of inferred losses being slightly higher on the phylogram (38·3). There are few inferred chromosome gains (phylogram 2·5; ultrametric tree 6·5) and demi-duplications (phylogram 8·9; ultrametric tree 5).

Inferred ancestral haploid chromosome numbers, which we refer to as *a*, are shown in the pie diagrams at the nodes of the trees. They were similar on the phylogram and ultrametric tree, with a few exceptions, mostly at deeper internal nodes where inferences had low statistical support [posterior probability (*PP*) <0·4; see legend in Fig. 1 and Supplementary Data Fig. S1]. Inference on the backbone was problematic for two nodes (marked with an asterisk in Fig. 1 and Supplementary Data Fig. S1) involving *Typhonium*, and *Theriophonum* for which an ancestral number of $a = 8$ has been inferred. These genera are embedded in clades with $a = 14$, which results in an inferred (but not statistically supported) decrease from $a = 14$ via 10 and 9, back to 14. Along the *Typhonium* backbone, the inferred ancestral haploid numbers decrease from $a = 8$ to 7, 6 and 5, with different states inferred for nodes in the *T. saraburiense*/*T. bulbiferum* clade on the phylogram and ultrametric tree (Fig. 1; Supplementary Data Fig. S1): on the ultrametric tree the inferred ancestral number for this clade is $a = 6$ (5) with the higher numbers ($n = 9$, 10) deriving from polyploidization events, and $n = 5$ in *T. baoshanense* and *T. jingpigense* being the ancestral condition. On the phylogram, the ancestral number is inferred as $a = 10$, with $n = 5$ the consequence of several chromosome losses. On both trees, other higher numbers, such as $n = 12$ in *T. circinnatum*, $n = 13$ in *T. roxburghii* and $n = 26$ in *T. blumei*, are inferred as resulting from polyploidization, while low numbers, such as $n = 4$ in *T.* spec. H.AR. 664 and in *T. laoticum*, are inferred as resulting from chromosome losses (descending dysploidy). Compared with the remaining Areae and the clade's outgroups, *Typhonium* has a low ancestral number ($a = 8$ or 7).

Molecular cytogenetic results

Observed chromosome numbers of the ten FISH-investigated species of *Typhonium* range from $2n = 8$ to $2n = 24$ (Table 2). They all have only one 5S rDNA site, with its distribution varying between species. In four species it was located interstitially, in five sub-terminally and in *T. laoticum* it had a proximal position (Figs 2 and 3B, E, H, K, N; Table 2). Most species had two 45S rDNA sites, predominantly distributed in terminal regions (Figs 2F, I, L, O and 3C, O). *Typhonium laoticum* ($2n = 9$) had a single 45S rDNA site, localized terminally on a chromosome pair (Fig. 3L), and *T. circinnatum* ($2n = 24$) had eight 45S rDNA sites located interstitially and/or terminally in eight chromosome pairs (Fig. 2C). *Typhonium huense* and *T. stigmatilobatum*, both with $2n = 15$, each had two 45S rDNA sites with an unusual number of signals (five; Fig. 3F, I; Table 2). The 5S and 45S rDNA sites were distributed on different chromosomes, with the exception of *T. circinnatum*, *T. huense* and *T. stigmatilobatum* (Figs 2B, C and 3E, F, H, I). rDNA satellites were seen in most cells (Figs 2L, O and 3F, L, O). For species on which no FISH experiments were performed, pictures of mitotic metaphases are provided in Supplementary Data Fig. S4.

Telomeric signals were localized at chromosome ends in all species. *Typhonium laoticum* in addition had two *Its* on its largest chromosome pair (Fig. 3J), and *Typhonium* spec. H.AR. 664 ($2n = 8$) had five *Its* positioned close to terminal regions on five chromosomes (Fig. 3M).

One small chromosome of the aneuploid species *T. trilobatum* (Fig. 2L, white arrowhead) yielded a diffuse rDNA signal, so we undertook additional experiments to find out the heterochromatin composition of this chromosome and if the diffuse 45S rDNA signal might be related to the amplification of one of its internal transcribed spacers. Similar experiments have been performed in plant species with B chromosomes (Dhar *et al.*, 2002; Marschner *et al.*, 2007). With C-banding (Fig. 4A, B), one chromosome was labelled along its length and was thus heterochromatic (Fig. 4B), while other chromosomes were labelled in sub-terminal or terminal regions. A *T. trilobatum*-specific ITS2 probe revealed only four signals (Fig. 4D) distributed in sub-terminal/terminal regions of a large and medium chromosome pairs. These sites represent the two rDNA sites seen in Fig. 2L.

DISCUSSION

Phylogenetic modelling of chromosome number change

With the current sampling of *Typhonium* (22 of its 50–60 species are included in our phylogeny) it appears that low chromosome numbers evolved twice, once in *T. baoshanense* and *T. jinpingense*, both with $2n = 10$ (Zhonglang *et al.*, 2002; Zhin-Lin *et al.*, 2007) and embedded among species with $2n = 18$–20, and again in *T. tubispathum* ($2n = 10$), *T. laoticum* ($2n = 9$) and *T.* spec. H.AR. 664 ($2n = 8$), which are embedded among species with $2n = 12$, 18 or 20. We believe that this inference is reliable because the tree is robust (nuclear and plastid regions were used; relevant nodes have good statistical support), and the key finding of a high dysploidy rate is insensitive to whether the inferences were made on a phylogram or on an ultrametric tree. How exactly branch lengths influence chromosome number reconstruction is currently not understood, and it is advisable to carry out maximum likelihood runs on both types of trees and then to trust those findings supported by both sets of reconstructions (Cusimano and Renner, 2014). Clearly, all character state reconstruction also stands and falls with dense species sampling and reliable counts for the included species. Regarding species sampling and chromosome counts in *Typhonium*, we have data for only about half the species in the genus. If the missing species had generally higher numbers, the inferred ancestral number in *Typhonium* might increase. However, the conclusion of at least two independent dysploidy events will not change by an improved sampling.

The main purpose of placing chromosome numbers in a phylogenetic context is to infer the likely direction of change, from high to low numbers or the other way around. While this is difficult to achieve, having an evolutionary framework is essential. Only cytogenetic methods, however, can then lead to an understanding of the mechanisms behind any inferred changes, and

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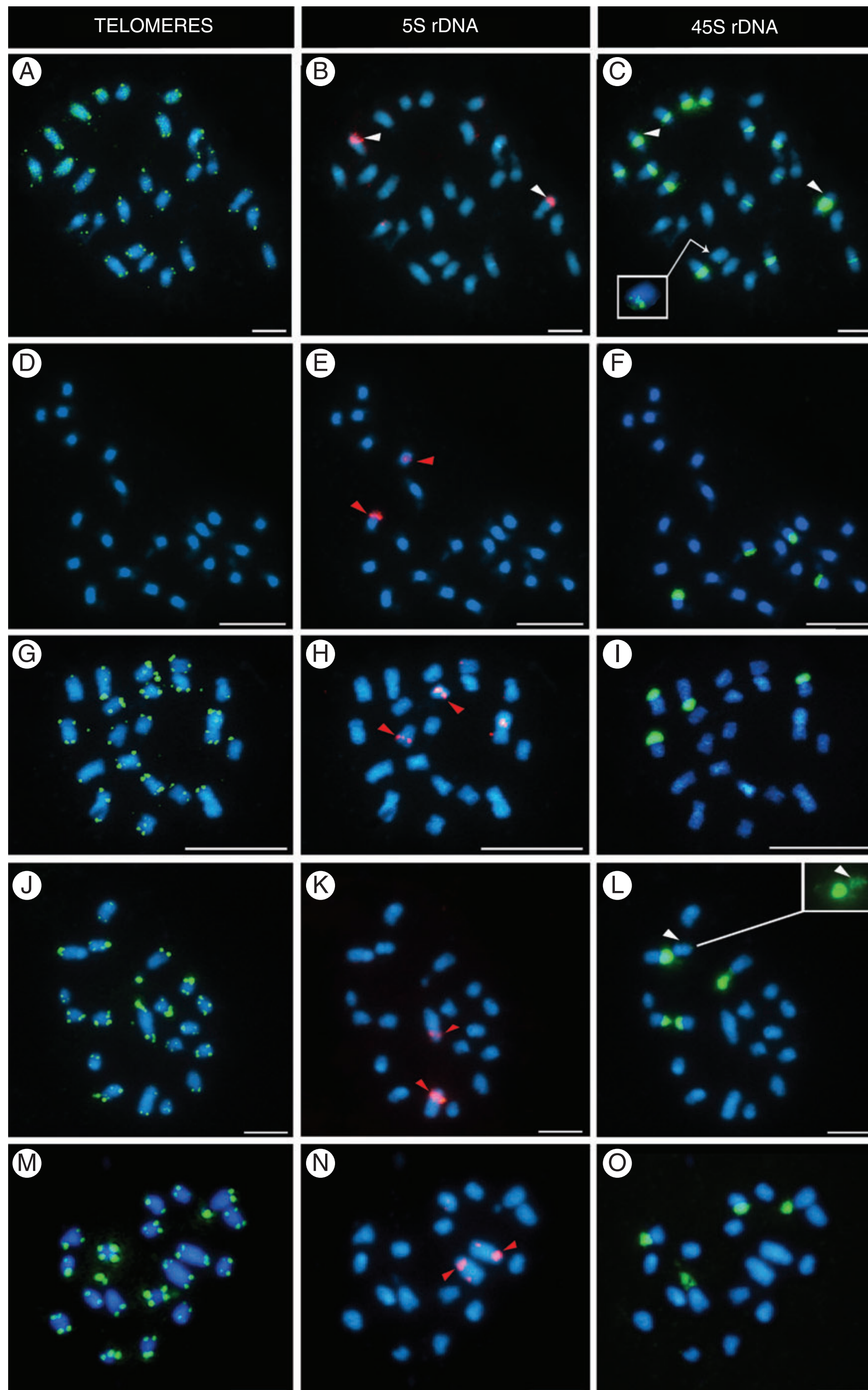

FIG. 2. Detection of telomeric signals, 5S and 45S rDNA sites in chromosomes of (A–C) *Typhonium circinnatum* (2*n* = 24), (D–F) *T. violifolium* (2*n* = 22), (G–I) *T. corrugatum* (2*n* = 20), (J–L) *T. trilobatum* (2*n* = 19) and (M–O) *T. saraburiense* (2*n* = 18) by FISH. Red arrowheads indicate the position of 5S rDNA sites in all cells, whereas white arrowheads in B and C indicate a chromosome pair with both rDNA sites, and in L a chromosome exhibiting a dispersed 45S rDNA signal. Insets in C show a chromosome with a weak 45S rDNA treated with a differential brightness/contrast, and in L a fifth diffuse 45S rDNA signal that overlaps the supernumerary chromosome. Scale bars = 5 µm.

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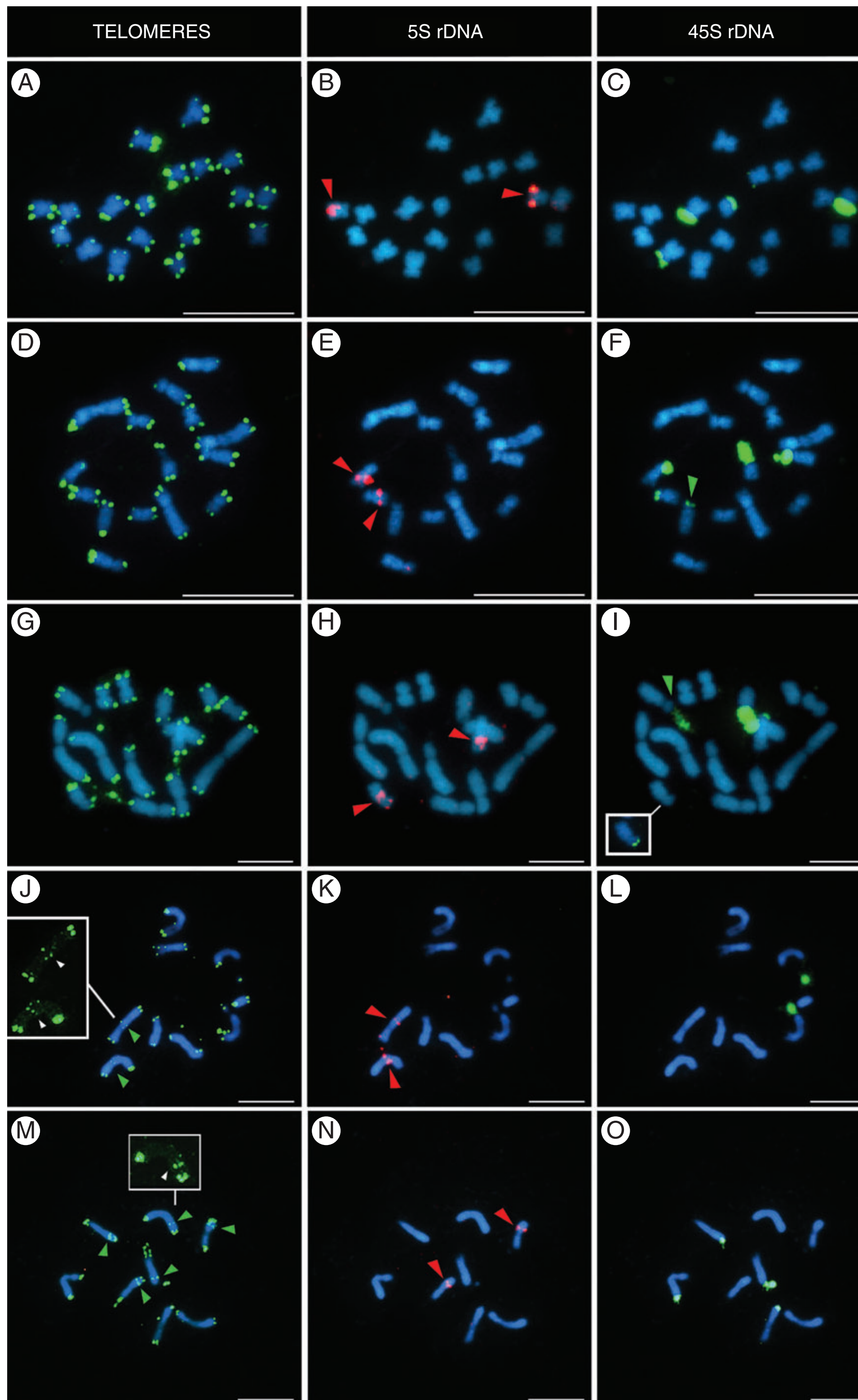

FIG. 3. Detection of telomeric signals, 5S and 45S rDNA sites in chromosomes of (A–C) *Typhonium echinulatum* ($2n = 18$), (D–F) *T. huense* ($2n = 15$), (G–I) *T. stigmatilobatum* ($2n = 15$), (J–L) *T. laoticum* ($2n = 9$) and (M–O) *T.* spec. H.AR. 664 ($2n = 8$) by FISH. Red arrowheads indicate the position of 5S rDNA sites in all cells, while green arrowheads in F and I indicate a fifth 45S rDNA signal and in J and M interstitial telomeric signals. Insets in I show chromosome with a weak 45S rDNA signal treated with a differential brightness/contrast, and in J and M display chromosomes with the telomeric probe, without the overlapping with DAPI. Scale bars = 5 µm.

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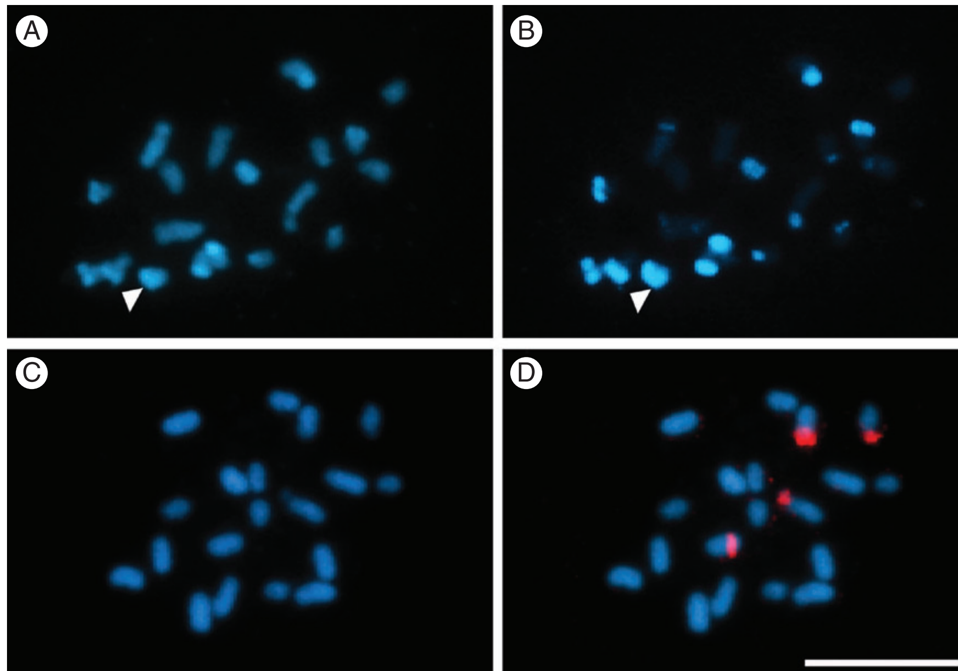

FIG. 4. Karyograms of *Typhonium trilobatum* ($2n = 19$). (A) Metaphase before and in (B) after C-banding. The heterochromatin is restricted mainly to sub-terminal/terminal regions of chromosomes, but only one chromosome (white arrowhead) was completely labelled and thus is heterochromatic. (C) Metaphase stained only with DAPI, and in (D) four signals visible after application of the *T. trilobatum* ITS2 probe. These signals correspond to the two sites of 45S rDNA. Scale bars = 5 μm.

full genome sequencing is required for detailed synteny. In this study, we brought together several of these approaches, using the same plant accessions, because we were initially critical of the high rate of chromosome 'loss' (decreasing dysploidy) inferred by the modelling approach.

Testing some of the inferred events with FISH

The FISH experiments, which to our knowledge are the first in the Araceae, revealed *Its* in the two *Typhonium* species with the lowest chromosome numbers, *T. laoticum* and *T.* spec. H.AR. 664. The ancestral state reconstructions (Fig. 1; Supplementary Data Fig. S1) for these species suggested number reduction by descending dysploidy. In other species with relatively low numbers, such as *T. stigmatilobatum* and *T. huense*, no dysploidy was inferred and no *Its* were detected. The cytogenetic evidence of *Its*, low chromosome numbers (incidentally including the lowest in the family) and aneuploid number series in combination suggests that dysploidy is an important mechanism in *Typhonium*. The consequences of dysploidy may include karyotype asymmetry and possibly also B chromosomes (Raskina *et al.*, 2008). Aneuploid numbers probably originate through meiotic irregularities leading to the formation of aneuploid gametes. Our *Typhonium* bulbs had been maintained in cultivation for several years, and, for each species, we had only one or a few individuals available for counting. Thus, the aneuploid chromosome numbers reported here may not represent the natural condition. It is also possible that some of the species are polyploids, suffering meiotic irregularities. So far, polyploidy had only been inferred for *T. trilobatum* and *T. roxburghii* (Cusimano *et al.*, 2012*a*; Supplementary Data Table S1), and we newly inferred it for *T. circinnatum* (see below).

How trustworthy are *Its* as indicators of evolutionary chromosome rearrangements (fusions) in *Typhonium*? Normally, telomeres protect chromosomes from end to end fusion (Slijepcevic, 1998), and their (rare) location in interstitial chromosome regions revealed in FISH studies is therefore interesting. Supplementary Data Fig. S5 illustrates the explanations proposed so far. Interstitial telomere signals have been related to paracentric or pericentric inversions, processes that do not imply a reduction in chromosome number (Supplementary Data Fig. S5a modified from Schubert, 2007). Another explanation for them is chromosome fusion by symmetrical reciprocal translocation involving the centromere (Supplementary Data Fig. S5b modified from Schubert and Lysak, 2011). This gives rise to a single chromosome and a small fragment composed mainly of the centromere of one chromosome and short rests of both previous chromosomes and their telomeres. Such short fragments will be eliminated from the cell unless they carry essential genes. A third mechanism, called a fusion–fission cycle or Robertsonian rearrangement, involves a reciprocal translocation with breakpoints within the telomeric arrays of two telocentric chromosomes. This preserves both chromosomes' centromeres and telomere sequences although one of the centromeres and the interstitial telomeric sequences must be inactive (Schubert and Lysak, 2011; Supplementary Data Fig. S5c). A large dicentric chromosome with/without *Its* may result, which can then break again and form two viable telocentric chromosomes (after formation of new telomeres). In plants, fusion–fission cycles have been documented in *Vicia faba* (Schubert *et al.*, 1995; Fuchs *et al.*, 1995: fig. 1). In *T. laoticum* and *T.* spec. H.AR. 664, however, we observed only one primary constriction, not two, which does not fit with a classical Robertsonian rearrangement.

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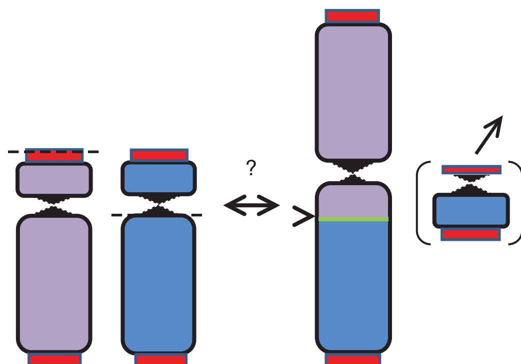
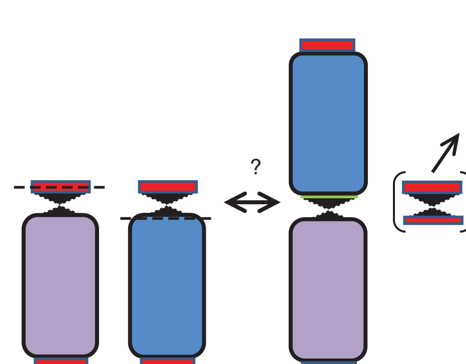
To explain the *Its* localized in the proximal region of the largest chromosome pair of *T. laoticum*, we now propose a new explanation (Fig. 5). It assumes a reciprocal translocation between two acrocentric chromosomes, with one chromosome having breaks in its telomere sequence array and the other having breaks close to the centromeric region of its long arm. The product of this translocation would be a submetacentric chromosome with a weakly detectible *Its*, no longer functional, plus a small chromosome comprising only part of the telomere sequence from one donor and the entire short arm and centromere of the other donor. Alternatively, a metacentric chromosome would be formed plus a small DNA fragment composed by only part of a telomere sequence from one donor and a centromere and complete telomere sequence array from the other donor (Fig. 5). We never found such small chromosomes, but the co-localization of *Its* with rDNA is suggestive. The presence of two *Its* in the proximal region of a large chromosome in *Sideritis montana* ($2n = 16$) has also been interpreted as indicating centric fusion and adduced to explain descending dysploidy (Raskina *et al.*, 2008).

To explain the *Its* close to the terminal regions of five chromosomes in *Typhonium* spec. H.AR. 664, we assume a mechanism similar to what has been suggested for *Pinus* (Schmidt *et al.*, 2000). Telomere-like repeats are highly amplified in *Pinus elliottii* and not restricted to the ends of chromosomes; instead they form large intercalary and pericentric blocks, attributed to random short sequence arrays, perhaps extended by slippage replication, insertion of extrachromosomal linear DNA fragments, or inversions (Biessmann and Mason, 1992). Meiotic studies would further clarify the pathways by which *T.* spec. H.AR. 664 (and also *T. laoticum*) acquired their low chromosome numbers. For example, a chromosome ring, as seen in *Eleocharis subarticulata* in meiosis I (Da Silva *et al.*, 2005), would point to multiple translocations having played a role in the reduction of chromosome number.

Polyploidy in T. circinnatum, *loss of a chromosome pair in* T. laoticum *and an rDNA cluster jump or amplification in* T. huense, T. stigmatilobatum *and* T. circinnatum

The 45S rDNA sites in *Typhonium* are stable in number and position (Table 2). Eight of the ten investigated species have two 45S rDNA sites, although *T. huense* and *T. stigmatilobatum* showed five instead of four signals at the two sites. Only *T. laoticum* has one site and *T. circinnatum* has eight rDNA





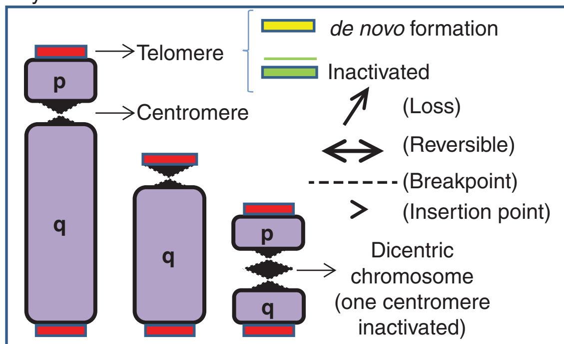


FIG. 5. Mechanisms explaining the interstitial telomeric signals on chromosomes of *Typhonium laoticum* (see text for details). Chromosome arms are labelled p for the short arm and q for the long arm. Telocentric chromosomes present only the long arm.

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sites. The increase of rDNA sites might indicate polyploidization, as indeed suggested by our chromosome number reconstruction for *T. circinnatum* (Fig. 1; Supplementary Data Fig. S1). Known polyploid angiosperms commonly show increased numbers of rDNA sites. Thus, in *Trifolium*, the allotetraploid *T. dubium* has twice the number the rDNA sites compared with any of its diploid parents, indicating additive inheritance (Ansari *et al.*, 2008). Also in *Aloe*, rDNA inheritance in polyploids is sometimes additive (Adams *et al.*, 2000). However, in the allotetraploid *Tragopogon mirus* and *T. miscellus*, both with $2n = 24$, the copy numbers of rDNA sites are slightly less than double the parental numbers (Kovarik *et al.*, 2005), while in intraspecific polyploids in *Passiflora*, rDNA site numbers exceed those expected under additive inheritance (De Melo and Guerra, 2003). Based on these examples, the eight rDNA sites of *Typhonium circinnatum* may well indicate a polyploidization event. Alternative explanations involve jumping nucleolus-organizing regions (Schubert and Wobus, 1985; for a review, see Raskina *et al.*, 2008), perhaps mediated by transposable elements. Such events could also explain the odd numbers of rDNA signals in *T. huense* (Fig. 3D–F) and *T. stigmatilobatum* (Fig. 3G–I). For *T. laoticum* (Fig. 3J–L), the loss of one chromosome pair with its rDNA site may explain the species' single 45S rDNA site.

B chromosomes in the Araceae – insufficiently tested so far

Supernumerary or putative B chromosomes have been reported from numerous species in seven genera of Araceae (*Anthurium*, *Apoballis*, *Arisaema*, *Asterostigma lividium*, *Philodendron radiatum*, *Piptospatha burbidgei* and *Schismatoglottis*), although not from *Typhonium* (original references in Supplementary Data table S1 in Cusimano *et al.*, 2012*a*). None of these studies used meiotic analyses for a more detailed understanding. Our C-banding and FISH experiments (using a specific ITS2 probe from *T. trilobatum*; Fig. 4A–D) appear to be the first molecular–cytogenetic analyses of any aneuploid chromosome number in the Araceae. The C-banding showed that heterochromatin blocks were mainly distributed in terminal regions of the regular chromosomes, while at least one small chromosome was completely stained (Fig. 4B). The complete staining resembles the situation in *Plantago lagopus* B chromosomes (Dhar *et al.*, 2002), a species in which the repetitive DNA of B chromosomes consists mainly of 5S rDNA (as shown with FISH). The small heterochromatic chromosome of *T. trilobatum* instead contained a single diffuse 45S rDNA signal (Fig. 2L, inset). Using the 18S nuclear ribosomal ITS2 of *T. trilobatum* as an *in situ* hybridization probe, we detected only four signals (Fig. 4D), representing the typical two 45S rDNA sites (Fig. 2L). These experiments, of course, are insufficient to establish the presence of B chromosomes, which can only be done by demonstrating meiotic drive in a population.

Conclusions

The new cytogenetic data supported two model-based inferences of descending dysploidy and one of polyploidization obtained in phylogenetic reconstructions of chromosome number change along a molecular phylogeny for *Typhonium* (using both phylograms and ultrametric trees). This is the first time that phylogenetic trait reconstruction for chromosome numbers has been tested by physical (microscopy-based) evidence. We also provide a detailed cytogenetic investigation of the aneuploid karyotype of *T. trilobatum*. The heterochromatic constitution of one of this species' chromosomes and the detection of dispersed 45S rDNA signals are reminiscent of B chromosomes in other plant species. However, without meiotic analyses, the existence of B chromosomes in the Araceae remains speculative.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: species and DNA regions sequenced, their sources and GenBank accession numbers. Figure S1: chromosome number reconstruction for the Areae on a phylogram, rooted on *Alocasia macrorrhizos*. Figure S2: maximum likelihood phylogeny for the Areae and three outgroups (*Alocasia*, *Arisaema* and *Pinellia*) based on the combined analysis of plastid and nuclear markers (4252 aligned nucleotides). Figure S3: chromosome number reconstruction for the Areae on an ultrametric tree rooted on *Alocasia macrorrhizos*. Figure S4: mitotic metaphases of *Typhonium filiforme*, *T. orbifolium*, *T.* spec. 17 Thailand and *T. gallowayi*, and karyogram of *T. tubispathum*. Figure S5: chromosome rearrangements that may lead to a reduction of chromosome numbers.

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SUPPLEMENTARY DATA

Table S1. Species and DNA regions sequenced, their sources, and GenBank accession numbers. BG stands for botanical garden, cult. for cultivated. Herbarium acronyms in parentheses are from Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp). Species used in the cytogenetic work are marked in bold.

Species and authors	Source	*Plastid trnK*	*Plastid rpl20-rps12*	*Nuclear PhyC*
Alocasia macrorrhizos (L.) Don	M. P. Medecilo 435 (De La Salle University, Dasmarinas Herbarium, Philippines, DLSU-DH)	JQ238841	JQ238925	JQ083523
Arisaema amurense Maxim.	J. Bogner 2759 (M)	-	AY248911	-
Arisaema balansae Engl.	D. K. Harder et al. 5739 (MO)	-	AY279139	-
Arisaema clavatum Buchet	G. Gusman 01084, cult.	-	AY279142	-
Arisaema concinnum Schott	W. Hetterscheid H.AR.313, cult.	-	AY279143	-
Arisaema costatum (Wall.) Mart.	W. Hetterscheid H.AR.287, cult.	-	AY279144	-
Arisaema dracontium (L.) Schott	T. Barkman 352 (WMU)	-	AY248914	-
Arisaema erubescens (Wall.) Schott	G. Gusman 99007, cult.	-	AY279146	-
Arisaema filiforme (Reinw.) Blume	G. Gusman 99084, cult.	-	AY279146	-
Arisaema flavum (Forssk.) Schott	YP-Q. Yang 1034 (KUN) (1); W. Hetterscheid s.n., 27.07.2001, cult. (2)	JF953250 (1)	AY248915 (2)	-
Arisaema flavum subsp. *flavum*	M. W. Chase 16880 (K)	-	AY376841	-
Arisaema flavum subsp. *tibeticum* J. Murata	A. M. Chambers s.n., 1.6.2002, China, Chusum, Tibet, wild collected	-	AY279150	-
Arisaema formosanum Hayata	G. Gusman 95173, cult.	-	AY279151	-
Arisaema franchetianum Engl.	M. W. Chase 10478 (K) (1); W. Hetterscheid s.n., 27.7.2001, cult. (2)	AM920628 (1)	AY279152 (2)	-
Arisaema heterophyllum Blume	G. Gusman 92100, cult.	-	AY248916	-
Arisaema ilanense J.C. Wang	J. C. Wang 11620 (TNU)	-	AY279155	-
Arisaema jacquemontii Blume	G. Gusman 96151, cult.	-	AY279156	-
Arisaema macrospathum Benth.	G. Gusman 97229, cult.	-	AY248917	-
Arisaema murrayi (Graham) Hook. ex Blatter	J. Murata 29 (TI)	-	AY279160	-
Arisaema negishii Makino	J. Murata s.n., 20.2.2002 (TI)	-	AY279161	-
Arisaema nepenthoides (Wall.) Mart.	B. W. Magrys s.n., 25.4.2002, cult.	-	AY279162	-
Arisaema rhizomatum C.E.C. Fisher	209-LSF-GBOWS 0218 (KUN) (1); B. Chen 06 (MO) (2)	JF953256 (1)	AY248919 (2)	

Arisaema ringens (Thunb.) Schott	G. Gusman 91250, cult.	-	AY279163	-
Arisaema serratum (Thunb.) Schott	T. Ohi-Toma Arisa222 (TI) (1); J. Murata 23-15 (TI) (2)	AB494679 (1)	AY279167 (2)	-
Arisaema speciosum (Wall.) Mart.	W. Hetterscheid H.AR.294, cult.	EU886502	AY279168	EU886470
Arisaema tortuosum (Wall.) Schott	P. Bruggeman, India, Anaimudi 20.5.2005 (M, photo voucher) (1) W. Hetterscheid s.n., 27.7.2002, cult. (2)	EU886577 (1)	AY248920 (2)	EU886469 (1)
Arisaema triphyllum (L.) Torr.	T. Barkman 351 (WMU)	-	AY248921	-
Arum apulum (Carano) P.C. Boyce	DNA bank 1022 (RBG Kew)	GU067591	-	-
Arum byzantinum Blume	D.C. Drummond 18, cult.	GU067593	-	-
Arum concinnatum Schott	B. W. Magrys s.n., 15.3.2002, cult.	EU886516	GU255991	-
Arum creticum Boiss. & Heldr.	H-J. Tillich 4881 (M)	EU886504	EU886595	-
Arum cylindraceum Gasp.	M. Neumann I 21/05, cult. BG Bonn	EU886511	-	-
Arum cyrenaicum Hruby	W. Lobin 6425 (BONN)	EU886515	EU886623	-
Arum dioscoridis Sm.	B. W. Magrys s.n., 15.3.2002, cult.	EU886505	GU255992	-
Arum elongatum Steven	DNA bank 12032 (RBG Kew)	GU067598	-	-
Arum euxinum R.R. Mill	DNA bank 11019 (RBG Kew)	GU067599	-	-
Arum hygrophilum Boiss.	W. Lobin 14469 (BONN)	EU886509	EU886620	EU886471
Arum idaeum Coustur. & Gandoger	J. Linz et al. 58, cult.	GU067602	-	-
Arum italicum Miller	Cult. BG Mainz, 20.7.2001	EU886517	AY248922	EU886472
Arum korolkowii Regel	S. Volz 20 (M)	EU886589	EU886598	-
Arum maculatum L.	N. Cusimano 06-3 (M, photo voucher)	EU886506	EU886593	-
Arum nigrum Schott	N. Cusimano 06-1 (M, photo voucher)	EU886507	EU886597	EU886473
Arum orientale Bieb.	Cult. BG Munich 06/1845w	EU886510	EU886621	-
Arum palaestinum Boiss.	J. Linz et al. 1, cult.	GU067607	-	-
Arum pictum L. f.	W. Lobin 273 (BONN)	EU886518	EU886596	-
Arum purpureospathum P.C. Boyce	E. Walton s.n., 15.4.2002, cult.	EU886508	EU886594	-
Arum rupicola Boiss.	J. Bogner 1790 (M)	EU886519	EU886592	-
Arum sintenisii (Engler) P.C. Boyce	D. C. Drummond 16, cult.	GU067613	-	-
Biarum bovei Blume	T. F. Hewer H1951 (M)	EU886529	EU886601	-
Biarum carduchorum (Schott) Engl.	M. Jaeger JLMS-60, cult. BG Giessen	EU886521	EU886618	-
Biarum davisii Turrill	Cult. BG Missouri, acc. 78231	EU886525	AY248923	EU886479-
Biarum dispar (Schott) Talavera	M. Jaeger SBL 564, cult. BG Giessen	EU886522	EU886619	-

Biarum ditschianum Bogner & Boyce	Cult. BG Bonn 4695	EU886526	EU886600	EU886477
Biarum kotschyi (Schott) B. Mathew ex H. Riedl	Cult. BG Bonn TR-0 BONN 8431	EU886527	EU886599	-
Biarum pyrami (Schott) Engler	J. Mayr s.n., cult. BG Giessen	EU886523	EU886617	-
Biarum tenuifolium (L.) Schott	Cult. BG Bonn 16014	EU886528	AY248924	-
Dracunculus canariensis Kunth	Cult. BG Bonn ES-0 BONN 13049	EU886531	AY248926	EU886475
Dracunculus vulgaris Schott	T. Croat 78286 (MO)	EU886532	AY248927	EU886476
Helicodiceros muscivorus (L. f.) Engl.	Cult. BG Missouri, acc. 71821	EU886533	AY248929	EU886480
Pinellia cordata N. E. Brown	J. McClements s.n., 30.7.2001, cult.	-	AY248930	-
Pinellia pedatisecta Schott	M. W. Chase 11752 (K) (1); J. McClements s.n., 30.7.2001, cult. (2)	AM920629 (1)	AY279170 (2)	-
Pinellia peltata (Thunb.) Breit.	J. W. Waddick s.n., cult. 8.8.2001	-	AY279171	-
Pinellia ternata (Thunb.) Breit.	J. McClements s.n., 30.7.2001	EU886503	AY248931	JQ083574
Pinellia tripartita (Blume) Schott	T. Ohi-Toma Pin02 (TI) (1); T. Croat 78128 (MO) (2)	AB494681 (1)	AY279172 (2)	-
Sauromatum diversifolium (Wall.) Cusimano & Hett.	W. Hetterscheid H.AR.484 (L, spirit coll.)	EU886540	EU886605	EU886482
Sauromatum gaoligongense Wang & H. Li	Y. M. Chen 24 (KUN)	EU886590	KC460384	EU886487
Sauromatum giganteum (Engl.) Cusimano & Hett.	J. W. Waddick s.n. 20.8.2001, cult.	EU886536	AY248938	EU886490
Sauromatum hirsutum (S. Y. Hu) Cusimano & Hett.	W. Hetterscheid H.AR.036 (L, spirit coll.)	EU886542	AY248939	EU886489
Sauromatum horsfieldii Miq.	J. Murata 3 (TI)	EU886541	EU886604	EU886483
Sauromatum tentaculatum (Hett.) Cusimano & Hett.	W. Hetterscheid H.AR.042 (L, spirit coll.)	EU886543	EU886612	EU886488
Sauromatum venosum (Dryand. ex Aiton) Kunth	J. Bogner 2972 (M)	EU886544	EU886603	EU886481
Theriophonum dalzellii Schott	P. Bruggeman PB168, India (M, photo voucher)	EU886534	KC460348	EU886486
Theriophonum infaustum N.E.Brown	P. Bruggeman PB099, India (M, photo voucher)	EU886535	EU886602	EU886485
Typhonium baoshanense Z.L. Dao & H. Li	Y. M. Chen 17 (KUN)	EU886591	EU886629	-
Typhonium blumei Nicholson & Sivadasan	G. Hausner 5 (M, photo voucher)	EU886553	KC460351	KC434103
Typhonium bulbiferum Dalzell	S. R. Yadav s.n., cult.	AB494517	AB494517	-
Typhonium circinnatum Hett. & J.Mood	W. Hetterscheid H.AR.248 (L, spirit coll.) = M. V. Silber 2 (M) from H.AR. 537	EU886551	-	-
Typhonium corrugatum Hett. & Rybkova	W. Hetterscheid H.AR.598, leg. R. Rybkova s.n., Vietnam = J. Bogner 2962 (M)	GU255984	-	KC434106
Typhonium echinulatum Hett. & Sookchaloem	W. Hetterscheid H.AR.225 (L, spirit coll.) = M. V. Silber 6 (M)	EU886554	KC460355	EU886499

Typhonium filiforme Ridl.	W. Hetterscheid H.AR.128 (L, spirit coll.)	EU886555	KC460356	KC434108
Typhonium flagelliforme (Lodd.) Blume	W. Hetterscheid H.AR.028 (L, spirit coll.)	EU886556	KC460357	-
Typhonium gallowayi Hett. & Sookchaloem	W. Hetterscheid H.AR.575 (L, spirit coll.) = A. Galloway AGA-1297-01	KC434090	KC460358	KC434109
Typhonium huense V.D. Nguyen & Croat	W. Hetterscheid H.AR.306 = M. V. Silber 11 (M, photo voucher)	KC434091	KC460362	KC434111
Typhonium H.AR. 523 spec. nov. Vietnam	W. Hetterscheid H.AR.523	KC434100	KC460378	KC434125
Typhonium jinpingense Z.L. Wang, H. Li & F.H. Bian	Y. M. Chen 023 (KUN)	EU886564	EU886614	EU886498
Typhonium laoticum Gagnep.	W. Hetterscheid H.AR.526 = M. V. Silber 8 (M)	KC434093	KC460364	KC434113
***Typhonium* spec.** H.AR. 664	W. Hetterscheid H.AR.664 = M. V. Silber 9 (M) = A. Galloway AGA-0521-01, collected on Doi Inthanon, Thailand	KC434089	KC460352	KC434104
Typhonium orbifolium Hett. & Sookchaloem	W. Hetterscheid H.AR.227 (L, spirit coll.)	EU886566	KC460366	KC478075
Typhonium roxburghii Schott	W. Hetterscheid H.AR.026	KC434095	-	KC434117
Typhonium roxburghii Schott	W. Hetterscheid H.AR.076	KC434094	KC460369	KC434116
Typhonium saraburiense Sookchaloem, Hett. & Murata	W. Hetterscheid H.AR.538 (L, spirit coll.) = A. Galloway AGA-1734-01, collected in Lop Bori, Thailand,	EU886570	KC460370	KC434118
***Typhonium* spec. 17**, Thailand	W. Hetterscheid H.AR.566 = M. V. Silber 7 (M) = A. Galloway AGA-1048-02, http://www4.ncsu.edu/~alan/plants/aroids/typhoniums/sp-017/	KC434098	KC460376	KC434123
Typhonium stigmatilobatum V.D.Nguyen	V. D. Nguyen 369 (HN)	KC434101	KC460379	KC434126
Typhonium trilobatum (L.) Schott	W. Hetterscheid s.n. = M. V. Silber 4 (M)	KC434102	KC460381	KC434127
Typhonium tubispathum Hett. & A.Galloway	W. Hetterscheid H.AR.469 (L, spirit coll.), CS-0201410, type, collected in Tak, Thailand	EU886574	KC460382	KC434128
Typhonium violifolium Gagnep.	W. Hetterscheid H.AR.168 (L, spirit coll.), Thailand	EU886562	EU886611	KC434129

Figure S1. Chromosome number reconstruction for the Areae on a phylogram, rooted on *Alocasia macrorrhizos*. Pie charts represent the probabilities of inferred chromosome numbers, with the number inside each pie having the highest probability. Numbers above branches are colour-coded by event type (gains, losses, duplications, demiduplication) as shown in the rectangular inset and represent the frequency with which event type(s) with a probability >0.5 occurred along that branch. The colour-coding of chromosome numbers is
explained in the elongated inset on the left. Problematic inferences on the backbone are marked with an asterisk. Species investigated by FISH are labelled in red while species which only chromosome counts were made are shown in blue.

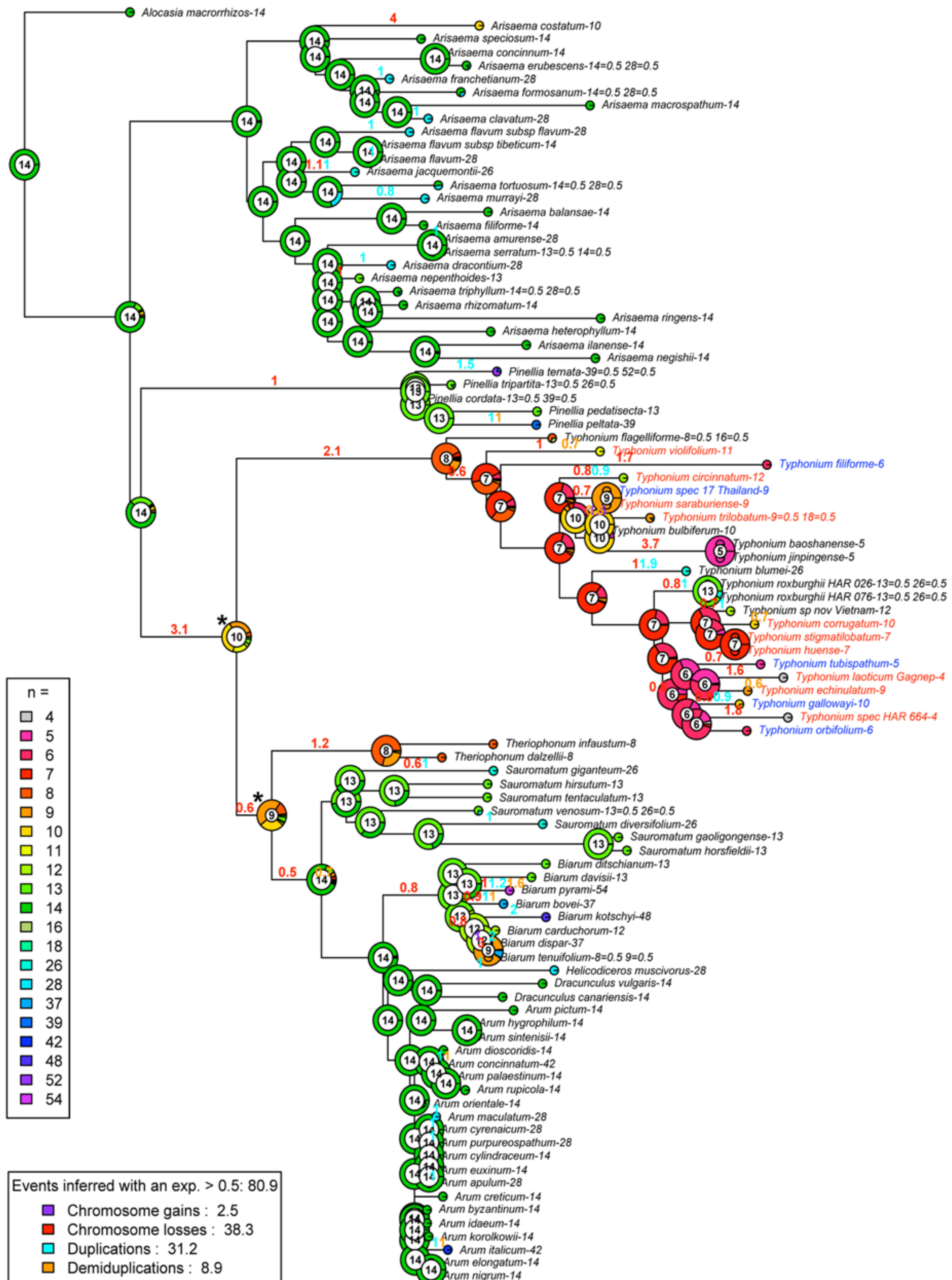


Figure S2. Maximum Likelihood phylogeny for the Areae and three outgroups (*Alocasia*, *Arisaema*, and *Pinellia*) based on the combined analysis of plastid and nuclear markers (4252 aligned nucleotides). The tree is rooted on *Alocasia macrorrhizos.* Bootstrap support (bold, above branch) and posterior probabilities (below branch) values are given at the nodes. Species investigated by FISH are labeled in red while species which only chromosome counts were made are shown in blue.

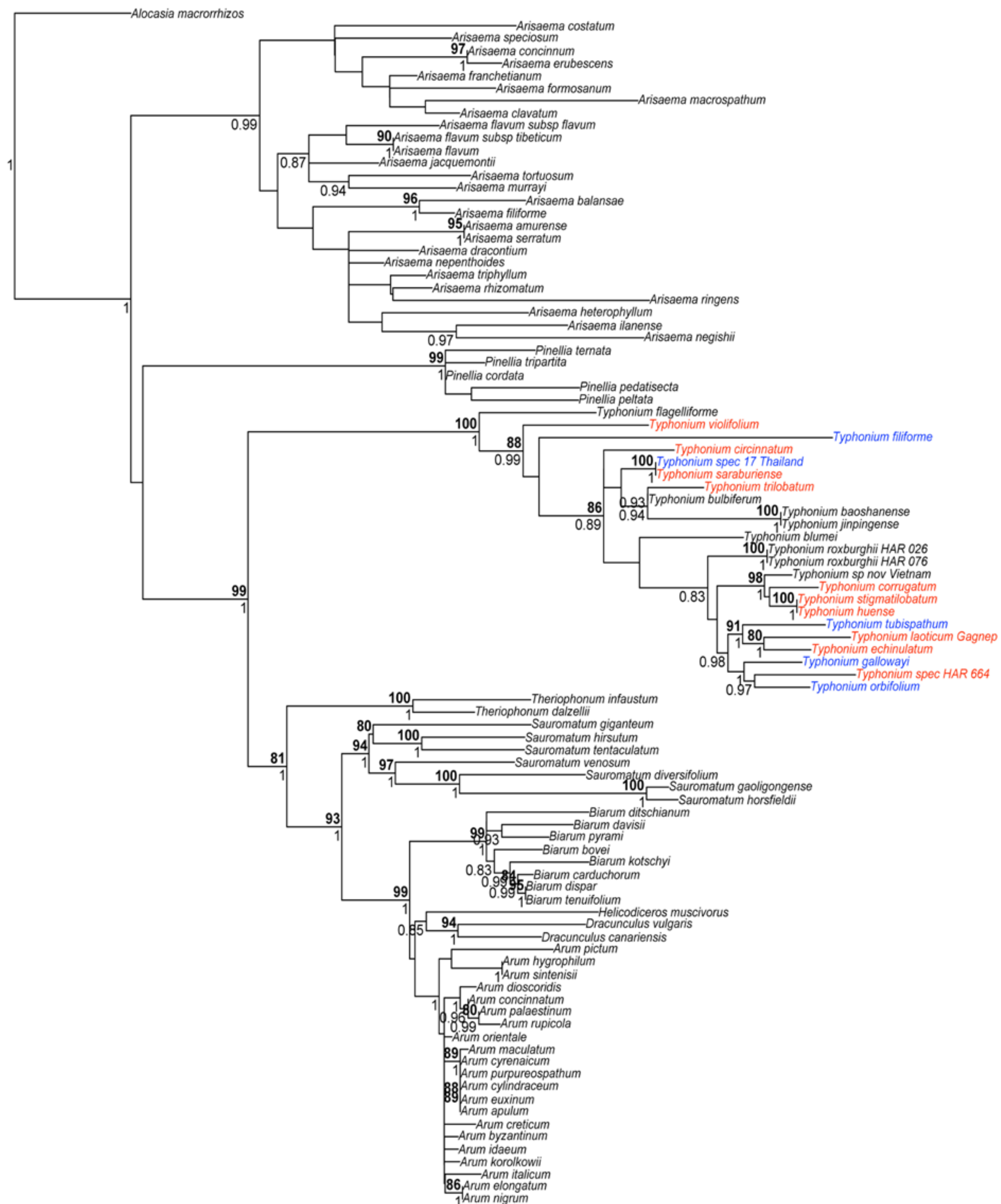


Figure S3. Chromosome number reconstruction for the Areae on an ultrametric tree rooted on *Alocasia macrorrhizos*. Posterior probabilities are indicated at nodes and the inferred frequency of the four possible events (gains, losses, duplications, demiduplications) with a probability >0.5 are shown above branches. The colour-coding of event types is explained in the inset. Species investigated by FISH are labeled in red while species which only chromosome counts were made are shown in blue.

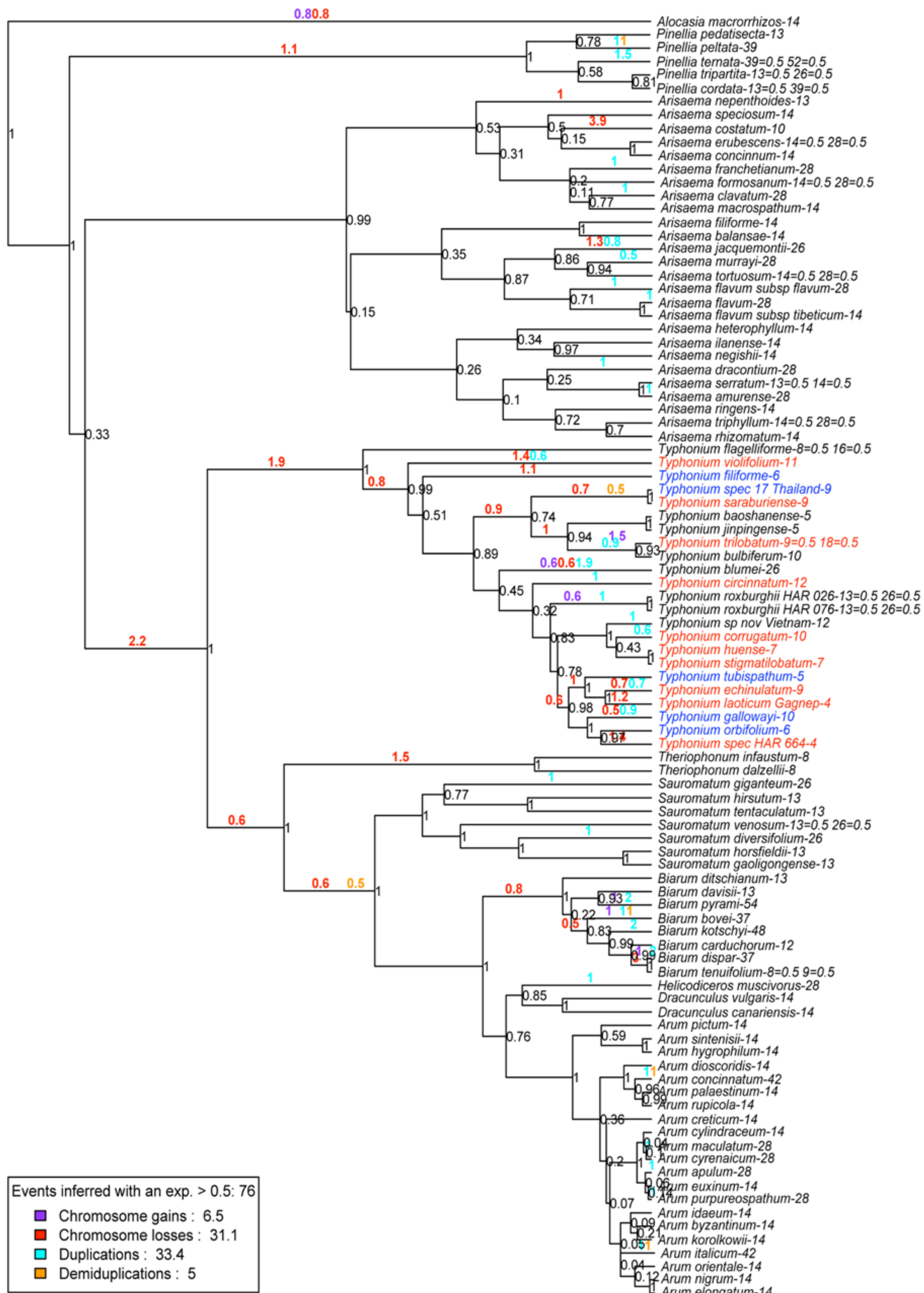


Figure S4. Mitotic metaphases of (A) *Typhonium filiforme* (2*n* = 12), (B) *T. orbifolium* (2*n* = 12), (C) *T.* spec. 17 Thailand (2*n* = 19), (D) *T. gallowayi* (2*n* = 20), and (E) karyogram of *T. tubispathum* (2*n* = 10). For (A), (B), and (D) photographs were scanned, and their chromosomes were counted using Adobe Photoshop CS3 version 10.0. All pictures were taken in a phase contrast microscope without staining, except by (C) which was stained in DAPI and photographed using a fluorescence microscope. Bars correspond to 5 µm.

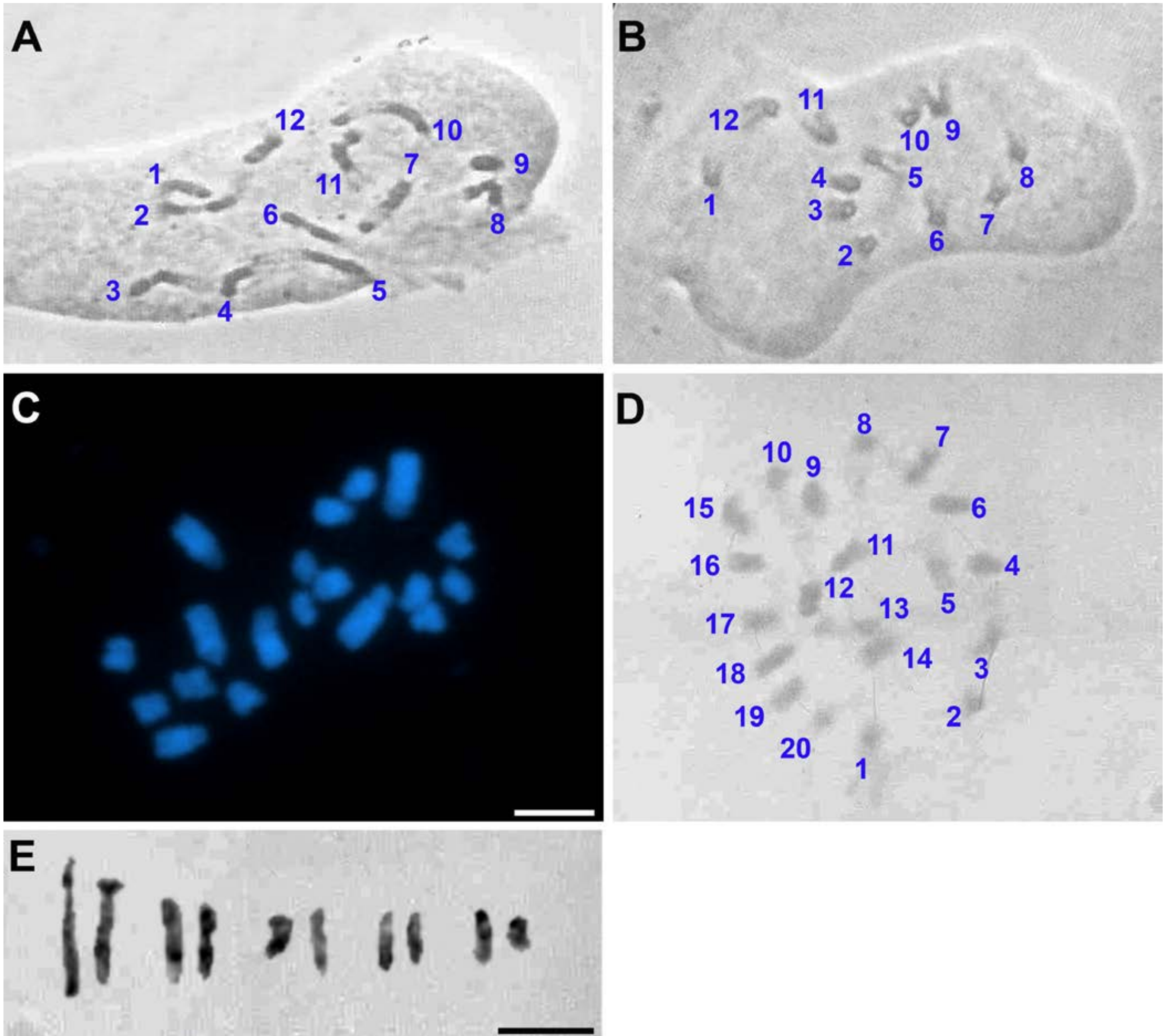
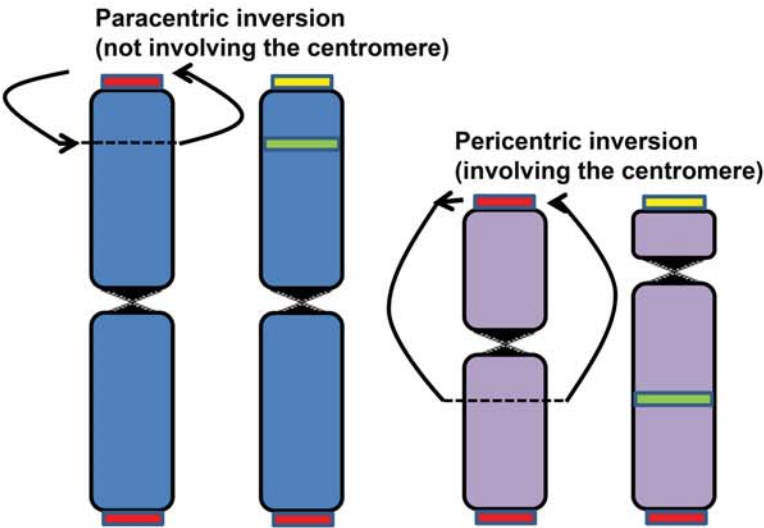
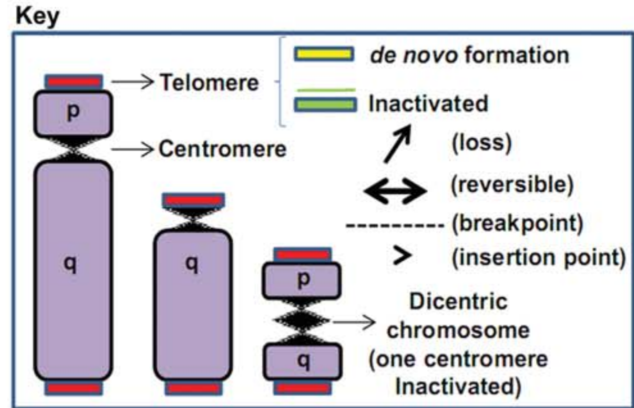


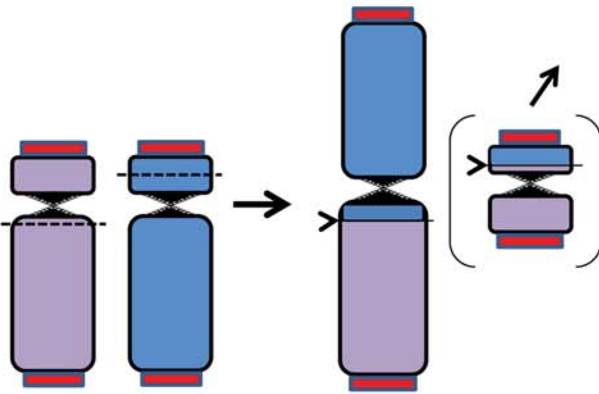
Figure S5. Chromosome rearrangements that may lead to a reduction of chromosome numbers. Chromosomes arms are labelled p for the short arm and q for the long arm. Telocentric chromosomes present only the long arm. Modified from Schubert (2007) and Schubert and Lysak (2011).

a) Interstitial telomeric sites as a result of chromosomal inversions





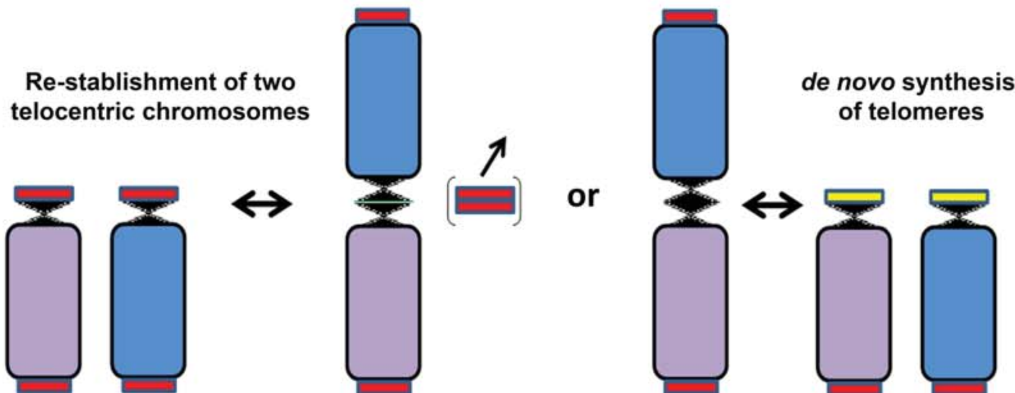
b) Chromosome fusion by symmetrical reciprocal translocation
(involving the centromere)



c) Fusion-fission cycle or Robertsonian rearrangements
Asymmetric reciprocal translocation
(not involving the centromere)

Formation of a dicentric chromosome that conserves some of its telomere

Dicentric chromosome without telomere sequences between centromeres



Chapter 4

Descending dysploidy, unusually large interstitial telomere bands, and chromosome evolution in the monocot family Araceae

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In review at *Molecular and Phylogenetic Evolution*

• Abstract

Chromosome losses and polyploidy appear to be the main evolutionary mechanisms generating chromosome number change, and both events can in principle be inferred on densely sampled phylogenies. Applied to the monocot family Araceae with chromosome numbers of $2n = 8$ to $2n = 160$, this type of approach has suggested that descending dysploidy has played a larger role than polyploidy in the evolution of the current chromosome numbers. Since dysploidy is commonly associated with interstitial telomeres, we carried out cytogenetic analyses of telomere organization in a sample of Araceae of pivotal phylogenetic position to search for possible interstitial telomeric signals. A phylogeny from plastid sequences for 174 species and new chromosome counts were used to newly model chromosome number evolution (in a maximum likelihood framework), and FISH with three probes (5S rDNA, 45S rDNA, and *Arabidopsis*-like telomeres) was performed on 14 species with $2n = 14$ to $2n = 60$. The chromosome number reconstruction on the phylogeny confirmed the large role of descending dysploidy in the Araceae. The number of 5S rDNA sites (one) was conserved, while the number of 45S rDNA sites varied from one to eight, and there was no correlation between the number of rDNA sites and ploidy level. Interstitial telomere repeats (ITRs) were detected in *Anthurium leuconerum*, *A. wendlingeri*, and *Spathyphyllum tenerum*, all with $2n = 30$. The ITR bands in *Anthurium* are of a type previously reported from the gymnosperms *Cycas* and *Pinus* and involve massive repeat amplification. Such extreme repeat amplification probably relates to transposable elements and chromosome rearrangements in driving Araceae genome evolution.

• Introduction

A phylogeny establishes the direction of evolution and allows reconstructing the likely timeframe and sequence of events that led to the character states seen in the included species. With the availability of DNA-based phylogenies, cytogeneticists have increasingly turned to “trait reconstruction” to infer the direction of change in chromosome numbers. Attention has mostly focusing on groups with polyploidy, while fewer studies have concentrated on clades with descending chromosome numbers (dysploidy), for example, in the Brassicaceae (Yogeeswaran et al., 2005; Lysak et al., 2006; Mandakova and Lysak, 2008; Cheng et al., 2013), Rosaceae (Vilanova et al., 2008; Illa et al., 2011; Jung et al., 2012), Poaceae (Luo et

al., 2009), and Melianthaceae (Pellicer et al., 2014). Probably the best studied case of chromosome rearrangements leading to descending dysploidy is *Arabidopsis*, where $n = 8$ is ancestral to $n = 5$ through inversions, fusions, and translocations (Lysak et al., 2006). This could be inferred by combining phylogenies for the relevant species with fluorescent *in situ* hybridization (FISH). Recent work in the large monocot family Araceae, with 3790 species in 118 genera (Boyce and Croat, 2011), revealed that in this family, too, dysploidy has played a much more important role than polyploidy (Cusimano et al., 2012: Table S1 lists all counts for the Araceae family; Sousa et al. in press). This inference, however, was based on a relatively sparse sample of species representing the many genera (Cusimano et al., 2012) and a follow-up study on a derived tribe, the Areae (Sousa et al., in press). The hypothesis of frequent chromosome losses in the Araceae therefore is in need of further cytogenetic testing.

One cytogenetic test for an inferred reduction in chromosome number is the presence of interstitial telomere repeats (ITRs), which can be visualized using standard probes for plant telomere repeats (Ijdo et al., 1991; Fuchs et al., 1995; Weiss-Schneeweiss et al., 2004). Such repeats may be found in interstitial positions because of translocations or inversions (Fuchs et al., 1995). They are also considered indicators of chromosome fusion. For example, telomere signals near a centromere may indicate the fusion of two telocentric chromosomes (Schubert, 1992). So far, *Pinus* is the genus with the most conspicuous interstitial telomere FISH signals, with often up to four signals near the centromere and in interstitial positions (Fuchs et al., 1995; Lubaretz et al., 1996; Schmidt et al., 2000; Hizume et al., 2002; Islam-Faridi et al., 2007). Based on the inferred large role of dysploidy in the Araceae (previous paragraph), we decided to carry out cytogenetic analyses of telomere organization, focusing on early-diverging genera in the Araceae phylogeny and on other genera of pivotal phylogenetic position. The only previous FISH study on the Araceae focused on species of *Typhonium*, a genus of Areae (Sousa et al., 2014). The enlarged Araceae phylogeny and new cytogenetic data on which we report here afford a better understanding of family-wide chromosomal patterns and the presence (or absence) of interstitial telomeric signals in the Araceae.

• Material and Methods

Plant material and DNA sequencing

We augmented the DNA data matrix of Nauheimer et al. (2012) by adding sequences for 29 further species from GenBank and by sequencing 14 additional species (on which cytogenetic studies were performed) for the same gene loci used by Nauheimer et al., viz. the plastid *trnL* intron and spacer, the *matK* gene and partial *trnK* intron, and the *rbcL* gene. We used standard primers, except for *matK* for which we used the primers listed in Cusimano et al. (2010). Total DNA from silica-dried leaves was extracted with the NucleoSpin plant II kit according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany). Polymerase chain reactions were performed using 1.25 units of Taq DNA polymerase (New England Biolabs GmbH, Frankfurt am Main, Germany) and the following cycle conditions: An initial step of 3 min at 95°C, followed by 39 cycles at 95°C for 30 sec for DNA denaturation, 60 sec at 50-52°C for primer annealing, 60 sec at 68°C for primer extension, and 10 min at 68°C after the final cycle. The PCR products were purified with Exo I and FastAP (Fermentas, St Leon-Rot, Germany). Sequencing was done on an ABI 3130 4-capillary sequencer, and sequences were assembled and edited with Sequencher 4.2 (Gene Codes Cooperation, Ann Arbor, Michigan, U.S.A.). The newly studied and sequenced species, with their taxonomic authors, herbarium vouchers, and GenBank accession numbers are listed in Supporting Information Table S1. For voucher information on the previously sequenced Araceae see Nauheimer et al. (2012; Table S1). The final alignment comprised 174: 163 Araceae plus 11 outgroups that represent the remaining families of the order Alismatales.

Phylogenetic analyses

Alignments were generated in MAFFT (Katoh and Standley, 2013; http://mafft.cbrc.jp/ alignment/server/) and checked visually using MEGA5 (Tamura et al., 2011). To remove poorly aligned positions, alignments were exported to a server runningGblocks v. 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with the least stringent options selected (Castresana, 2000). The combined matrix (4928 aligned nucleotides) was used for maximum likelihood (ML) tree searches in RAxML (Stamatakis, 2006; Stamatakis et al., 2008), using the GTR + G substitution model with four rate categories. Bootstrapping under ML used 1000 replicates. We also generated ultrametric trees in BEAST v. 1.7.5 (Drummond and Rambaut, 2007), using the same substitution model for

the entire concatenated alignment and a pure-birth Yule model as the tree prior. The analysis was run for 100 million generations, sampling every 1000th step. The burn-in fraction, i.e., the number of trees to be discarded before constructing a consensus tree (the maximum clade credibility tree) from the remaining trees, was assessed using Tracer v. 1.4.1, which is part of the BEAST package.

Inference of chromosome number change

For maximum likelihood and Bayesian phylogenetic inferences of ancestral haploid chromosome numbers we used ChromEvol v. 1.4 version with eight models (Mayrose et al., 2010; http://www.tau.ac.il/~itaymay/cp/chromEvol/index.html). ChromEvol models change in chromosome number with the following parameters: polyploidization (chromosome number duplication) with constant rate ρ, demi-duplication (fusion of gametes of different ploidy) with constant rate μ, and dysploidization with either constant or linearly changing rates (ascending: chromosome gain rates λ or λ_1; descending: chromosome loss rates δ or δ_1). We fitted all models to a phylogram (in which branch lengths are proportional to numbers of substitution) and an ultrametric depiction of the phylogeny (in which branch lengths are proportional to time). The ultrametric tree was the BEAST maximum clade probability tree. For each model, we ran 10000 simulated repetitions to compute the expected number of changes along each branch of the phylogeny as well as the ancestral haploid chromosome numbers at nodes. The maximum possible ancestral number of chromosomes was set to 10x higher than the highest number found in the empirical data, the minimum number was set to 1. Species' haploid chromosome numbers were obtained from Cusimano et al. (2012, Supplementary Data Table S1) and from the Index to Plant Chromosome Numbers (http://www.tropicos.org/Project/IPCN); species without known numbers were coded as 'unknown' (X), and changes among character states (chromosome numbers) were assigned equal likelihood. Model fit was assessed via likelihood ratio tests using the Akaike information criterion (AIC). We adjusted the phylogram and the ultrametric tree such that both had a total length of 0.2. Results were plotted in R using the ChromEvol functions version 1 of N. Cusimano (http://www.sysbot.biologie.uni-muenchen.de/en/people/cusimano/).

Chromosome preparation, FISH analyses, and DNA probes

Root tips were collected from potted plants cultivated in the greenhouses of the Munich Botanical Garden. Authors of species names and voucher material for each species are given in Table S1. Root tips were pre-treated in 2 mM 8-hydroxyquinoline for 20 h at 4°C, fixed in freshly prepared 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and kept at -20°C. For chromosome preparations, fixed root tips were washed three times for 5 min in distilled water, digested with 1% cellulase (w/v; Onozuka RS, Serva), 0.4% pectolyase (w/v; Sigma), 0.4% cytohelicase (w/v; Sigma) in citric buffer, pH 4.8 for 30 min at 37°C in a humid chamber, dissected in a drop of 45% acetic acid and squashed. Coverslips were removed after freezing in dry ice, and preparations were air-dried at room temperature. The quality of spreads was checked microscopically using phase-contrast, and only preparations with at least 10 well-spread metaphases were used for FISH.

We performed FISH with probes for telomere repeats, 5S rDNA, and 45S rDNA. For some species, we had little material and could only use one or two of the three probes. To locate the rDNAs, we used the 18S-5.8S-25S rDNA repeat unit of *Arabidopsis thaliana* in the pBSK+ plasmid, labeled with digoxigenin-11-dUTP (Roche) by nick translation, and a 349-bp fragment of the 5S rRNA gene repeat unit from *Beta vulgaris* cloned into pBSK+ (Schmidt et al., 1994) and labeled with biotin-16-dUTP (Roche) by PCR. Telomere repeats were visualized with the *Arabidopsis*-like telomere probe of Ijdo et al. (1991) using the oligomer primers (5´-TTTAGGG-3´)$_5$ and (5´-CCCTAAA-3´)$_5$, labeled with digoxigenin-11-dUTP by nick translation. Hybridization mixes consisted of 50% formamide (w/v), 2 x SSC, 10% dextran sulfate (w/v), and 70–200 ng of labeled probe. The hybridization mix was denatured at 75ºC for 10 min and immediately cooled on ice for 10 min; 10–15 µl of the mix was then added to each slide. Hybridization was carried out in a humid chamber at 37ºC for 20 h. The 5S rDNA was detected with streptavidin-Cy3 conjugate (Sigma), and the 45S rDNA with anti-DIG-FITC conjugate (Roche) at 37ºC for 1 h. The chromosomes were counterstained with DAPI (2 µg/ml) and mounted in Vectashield (Vector).

Slides were first analyzed with the probes for telomeres and 5S rDNA, then de-stained, and then analyzed with the probe for the 45S rDNA. For some species with multiple 45S rDNA sites or with interstitial telomere repeats, further single-probe experiments were carried out to confirm the number of signals. Images were taken with a Leica DMR microscope equipped with a KAPPA-CCD camera and the KAPPA software. They were optimized for

best contrast and brightness using Adobe Photoshop CS3 version 10.0.

• Results

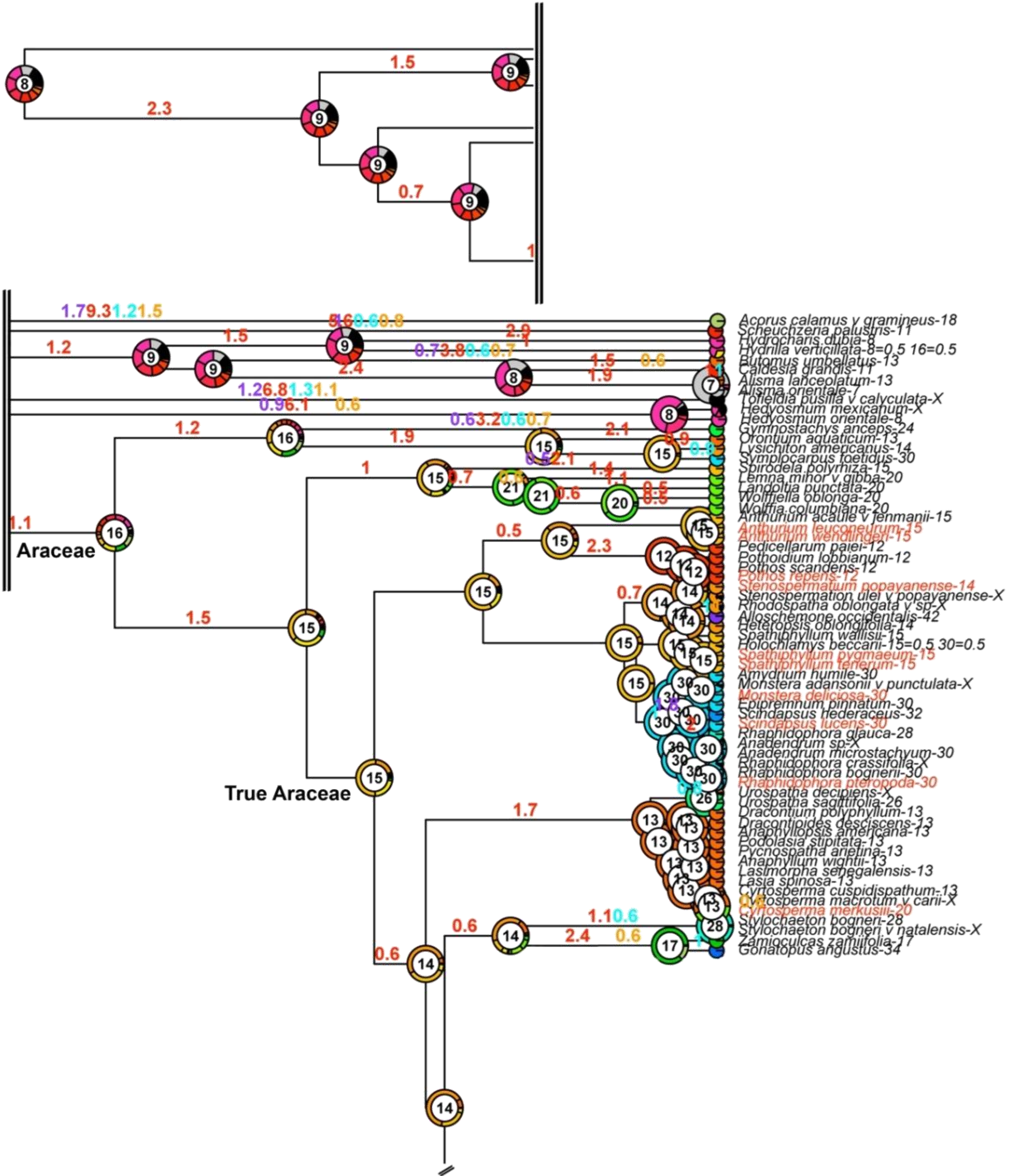
Chromosomal evolution in the Araceae

The plastid DNA matrix of 4928 aligned nucleotides for 174 species yielded a well-supported maximum likelihood phylogeny that we used to infer the evolution of Araceae chromosome numbers (Figs. 1 and S1). The changes inferred on the ultrametric Araceae tree are shown in Fig. 1, a reconstruction on the phylogram in Fig. S1. The statistical support for both trees is shown in Figs. S2 and S3, and the inferred rates of change and numbers of events are summarized in Table 1. On the ultrametric tree, the four-parameter-constant-rate model, which assumes constant chromosome gain and loss rates and a polyploidization rate that differs from the demi-polyploidization rate, best explained the data (AIC = 732.6 compared to 736.6 for the next best model), while on the phylogram, the best model was the four-parameter-linear-rate model, which includes rates of gain and loss that depend linearly on the current chromosome number (AIC = 844.4 compared to 982.8 for the next best). In both trees, chromosomes loss was the most common event. On the ultrametric tree, the next most common events were duplication of the entire chromosome complement and demi-duplications (Fig. 1 insets in the lower left, Table 1); on the phylogram, the next most common events were single chromosome gains, duplication of the entire set, and demi-duplications (Fig. S1 insets in the lower left, Table 1). The inferred ancestral haploid numbers in the Araceae decrease from a = 16 to 15 and 14 on the ultrametric tree and from a = 16 to 14 and 13 (and then back to a = 14) on the phylogram.

Table 1 Inferred chromosome number evolution in Araceae and their immediate outgroups under the best-fitting model. Column two refers to the factor used to multiply branch lengths to obtain a suitable root-to-tip length for the tree (Materials and Methods); columns three and four give the lengths obtained after adjusting branch lengths by the multiplication factor; column five gives the logarithmic likelihood, and column six the AIC scores to the likelihood ratio tests; the symbols for the rates inferred for all events in the tree are λ: chromosome gain rate; δ: chromosome loss rate; ρ: duplication rate; μ: demiduplication rate, and the number of events refers to the four event types with an expectation >0.5 (demi.: demiduplication). The last column shows the total number of events inferred on the respective tree.

							Rates				Number of Events				
Tree	Factor	Total tree length	Root-tip length	**Best model**	**LogLik**	**AIC**	λ	δ	ρ	μ	**Gains**	**Losses**	**Duplications**	**Demi.**	**Total events**
Ultrametric	0.3	3.6	0.75	crde	-362.3	732.6	6.96	43.67	8.28	7.83	9.1	135.6	21.2	17.8	183.7
Phylogram	0.2	1.5	0.9	lrde	-416.2	844.4	0	75.11	18.67	15.78	35.4	133.1	18.4	15.8	202.7

Fig. 1 (***facing page***) Chromosome number reconstruction for Araceae on an ultrametric tree, rooted on *Acorus calamus*. Pie charts represent the probabilities of inferred chromosome numbers, with the number inside a pie having the highest probability. Numbers above branches are color-coded by event type (gains, losses, duplications, demiduplication) as shown in the rectangular inset and represent the frequency with which event type(s) with a probability >0.5 occurred along that branch. The color-coding of chromosome numbers is explained in the inset on the left. Species investigated by FISH are labeled in red.


Araceae
True Araceae
Acorus calamus v gramineus-18
Scheuchzeria palustris-11
Hydrocharis dubia-8
Hydrilla verticillata-8=0.5 16=0.5
Butomus umbellatus-13
Caldesia grandis-11
Alisma lanceolatum-13
Alisma orientale-7
Tofieldia pusilla v calyculata-X
Hedyosmum mexicanum-X
Hedyosmum orientale-8
Gymnostachys anceps-24
Orontium aquaticum-13
Lysichiton americanus-14
Symplocarpus foetidus-30
Spirodela polyrhiza-15
Lemna minor v gibba-20
Landoltia punctata-20
Wolffiella oblonga-20
Wolffia columbiana-20
Anthurium acaule v jenmanii-15
Anthurium leuconeurum-15
Anthurium wendlingeri-15
Pedicellarum paiei-12
Pothoidium lobbianum-12
Pothos scandens-12
Pothos repens-12
Stenospermation popayanense-14
Stenospermation ulei v popayanense-X
Rhodospatha oblongata v sp-X
Alloschemone occidentalis-42
Heteropsis oblongifolia-14
Spathiphyllum wallisii-15
Holochlamys beccarii-15=0.5 30=0.5
Spathiphyllum pygmaeum-15
Spathiphyllum tenerum-15
Amydrium humile-30
Monstera adansonii v punctulata-X
Monstera deliciosa-30
Epipremnum pinnatum-30
Scindapsus hederaceus-32
Scindapsus lucens-30
Rhaphidophora glauca-28
Anadendrum sp-X
Anadendrum microstachyum-30
Rhaphidophora crassifolia-X
Rhaphidophora bognerii-30
Rhaphidophora pteropoda-30
Urospatha decipiens-X
Urospatha sagittifolia-26
Dracontium polyphyllum-13
Dracontioides desciscens-13
Anaphyllopsis americana-13
Podolasia stipitata-13
Pycnospatha arietina-13
Anaphyllum wightii-13
Lasimorpha senegalensis-13
Lasia spinosa-13
Cyrtosperma cuspidispathum-13
Cyrtosperma macrotum v carii-X
Cyrtosperma merkusii-20
Stylochaeton bogneri-28
Stylochaeton bogneri v natalensis-X
Zamioculcas zamiifolia-17
Gonatopus angustus-34

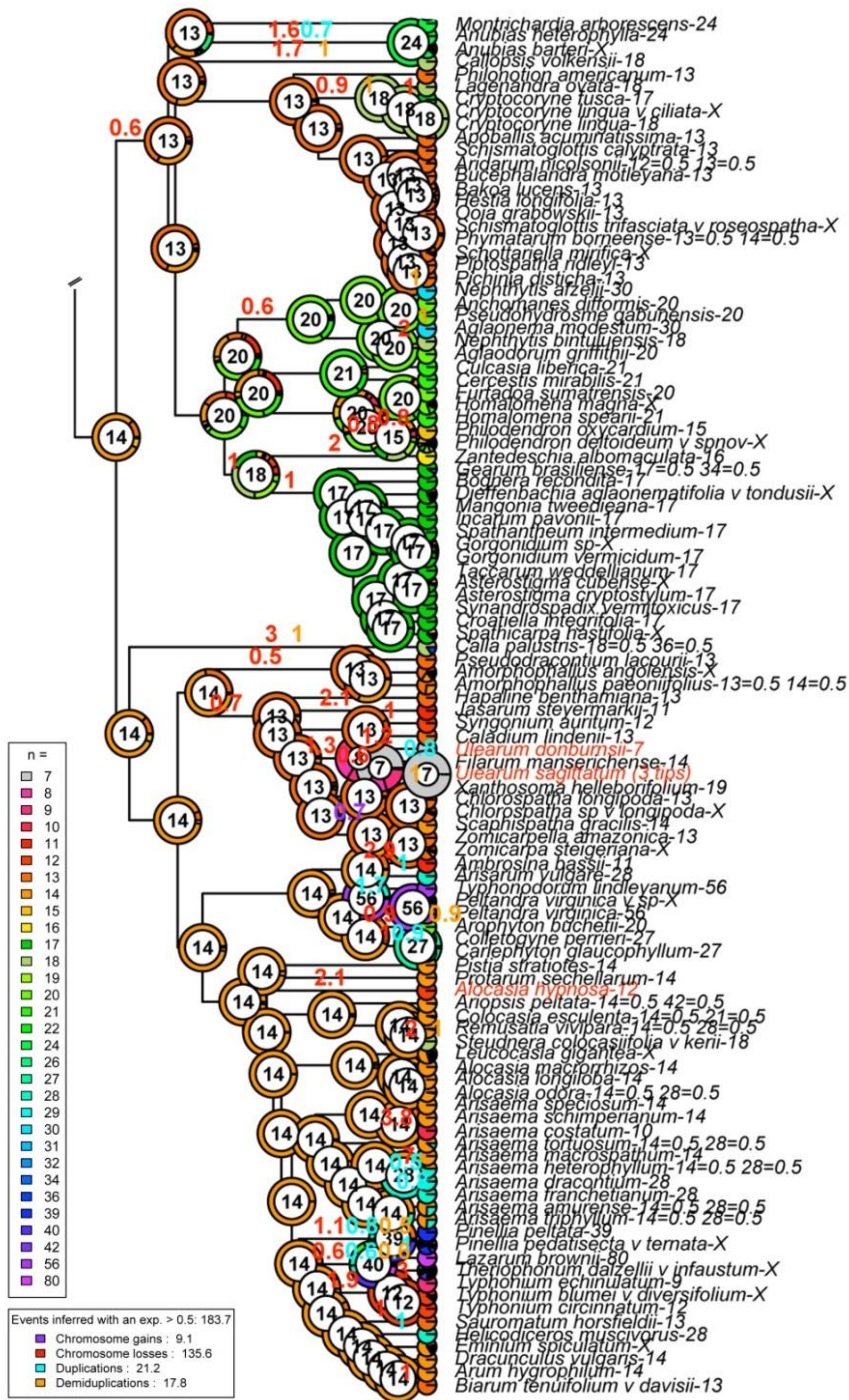


Fig. 1 (*continuation*)

Fluorescence *in situ* hybridization (FISH) experiments

Fluorescence *in situ* hybridization was performed in 14 species from 11 genera representing early and derived lineages of the Araceae (Table 2). We also here report new chromosome numbers for *Cyrtosperma merkusii*, *Pothos repens, Spathiphyllum pygmaeum*, *S. tenerum*, *Stylochaeton puberulus*, and *Ulearum sagittatum*. Their somatic numbers varied from 2*n* = 14 to 2*n* = 60 (Table 2). The chromosome variation found within each genus is presented in Table S2. *Ulearum*, with 2*n* = 14, has especially large chromosomes (Fig. 2a, d). The remaining species with relatively high chromosome numbers (2*n* = 24, 26, 28 and 30) have large or medium-sized chromosomes (Figs. 2, 3, and S4); species with 2*n* = 60 all have medium to small chromosomes (Figs. 3 and S4).

Of the 12 species tested with the 5S rDNA probe, six had one interstitial site, five had one sub-terminal site (Fig. 2b, e, h, k, n; Fig. 3b, e, h, k; Fig. S4b, e; Table 2), and one (*Anthurium wendlingeri*) yielded no signal. In *Rhaphidophora pteropoda*, one 5S rDNA signal was detected on a single chromosome in some cells but its homologous pair was never seen (data not shown). In *Cyrtosperma merkusii*, with 2*n* = 39, and in *Englerarum hypnosum* (the former *Alocasia hypnosa*; Nauheimer and Boyce, 2013), with 2*n* = 24, three instead of two 5S rDNA signals were detected (Figs. 3h and S4e).

Among the 11 species tested with the 45S rDNA probe, some had one, others up to eight sites, without any correlation between species' chromosome numbers and 45S site numbers. For example, *Ulearum donburgii* and *U. sagittatum*, both with *2n* = 14, had two sites just like *Anthurium leuconerum* with 2*n* = 30 (Fig. 2c, f, o), and *Scindapsus lucens* and *Rhaphidophora pteropoda,* both with 2*n* = 60 (Fig. S4c, i). The localization of 45S rDNA sites varied from terminal and subterminal to interstitial (Table 2; for lack of material, no experiments could be performed on *Englerarum hypnosum* and no 45S signal was found in *Anthurium wendlingeri*). In *Cyrtosperma merkusii*, three 45S rDNA signals were detected (Fig. 3i), the same number as that species' 5S rDNA signals (Fig. 3h). Especially high numbers of 45S rDNA were found in *Spathiphyllum* (Fig. 3c, f), distributed close to, or inside, the pericentric region (DAPI-positive repetitive DNA). The distribution of 45S in *Ulearum* and *Anthurium* was similar, but their centromeric regions were not DAPI positive (Fig. 2c, f, o; Table 2).

Of the 13 species tested with the telomere probe, all had telomeric signals at both chromosome ends (Fig. 2a, d, j; Fig. 3a, g, j; Fig. S4a, d, f, h), and three had additional

interstitial telomeric signals, namely *Anthurium leuconerum*, *A. wendlingeri*, and *Spathiphyllum tenerum*, with 12, multiple, or four interstitial signals localized in pericentric regions (Figs. 2m, 3d, and S4g).

Table 2 Araceae species investigated with their chromosome number, presence of interstitial telomere repeats (ITRs), and the number and distribution of 5S and 45S rDNA sites. Authors of species names and voucher information are given in Table S1. Asterisks mark species for which chromosome counts were newly obtained. X indicates species where the hybridization did not work or the pattern was not clear, hence the question mark. The symbol ∞ means multiple signals, and ♦ means that no ITRs were seen. NA = non applicable.

Species	2*n*	ITRs	# 5S rDNA	Distribution	# 45S rDNA	Distribution
Anthurium leuconerum	30	12	1	Subterminal	2	Pericentromeric
Anthurium wendlingeri	30	∞	X	?	X	?
*Cyrtosperma merkusii**	39	-	1(3)	Subterminal	1(3)	Terminal
Englerarum hypnosum	24	-	1(3)	Interstitial	NA	NA
Monstera deliciosa	60	-	1	Interstitial	1	Terminal
Rhaphidophora pteropoda	60	-	X	?	2	Terminal
Scindapsus lucens	60	-	1	Subterminal	2	Terminal
*Spathiphyllum pygmaeum**	30	-	1	Subterminal	3	Interstitial
*Spathiphyllum tenerum**	30	4	1	Subterminal	8	Interstitial
Stenospermatium papayanense	28	-	1	Interstitial	1	Terminal
*Stylochaeton puberulus**	26	♦	1	Interstitial	1	Terminal
Ulearum donburgii	14	-	1	Interstitial	2	Pericentromeric
*Ulearum sagittatum**	14	-	1	Interstitial	2	Pericentromeric
*Pothos repens**	24	-	NA	NA	NA	NA

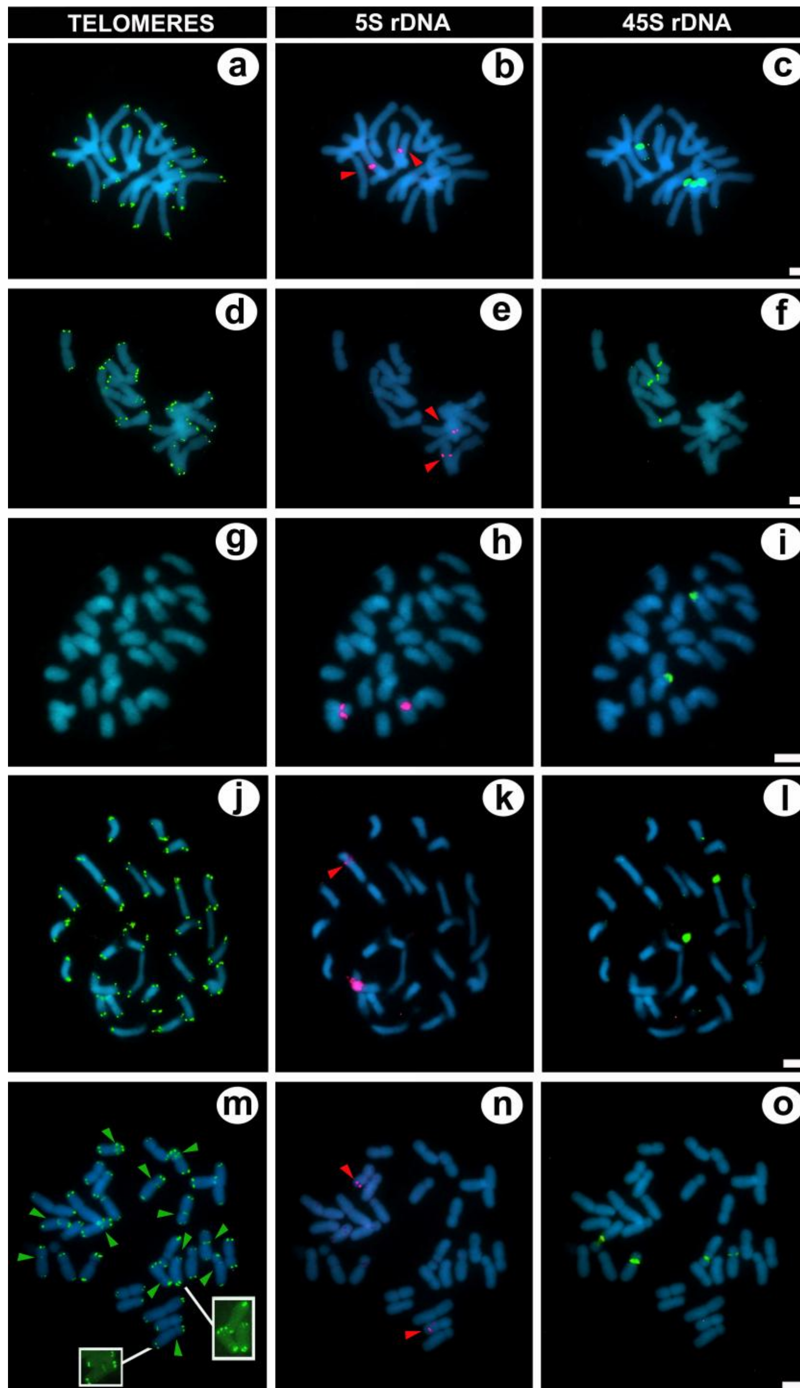


Fig. 2 Detection of telomeric signals, 5S and 45S rDNA sites in chromosomes of (a–c) *Ulearum donburgii* (2*n* = 14), (d–f) *Ulearum sagittatum* (2*n* = 14), (g–i) *Stylochaeton puberulus* (2*n* = 26), (j–l) *Stenospermatium papayanense* (2*n* = 28), and (m–o) *Anthurium leuconerum* (2*n* = 30) by FISH. The detection of the telomeres was not performed in *Stylochaeton puberulus*. Red arrowheads indicate the position of weak 5S rDNA sites in some cells, while green ones in (m) indicate ITRs. Insets in (m) show chromosomes, without being overlapped with DAPI, with weak ITRs treated with a differential brightness/contrast. Bars correspond to 5 μm, and are valid for plates in each row.

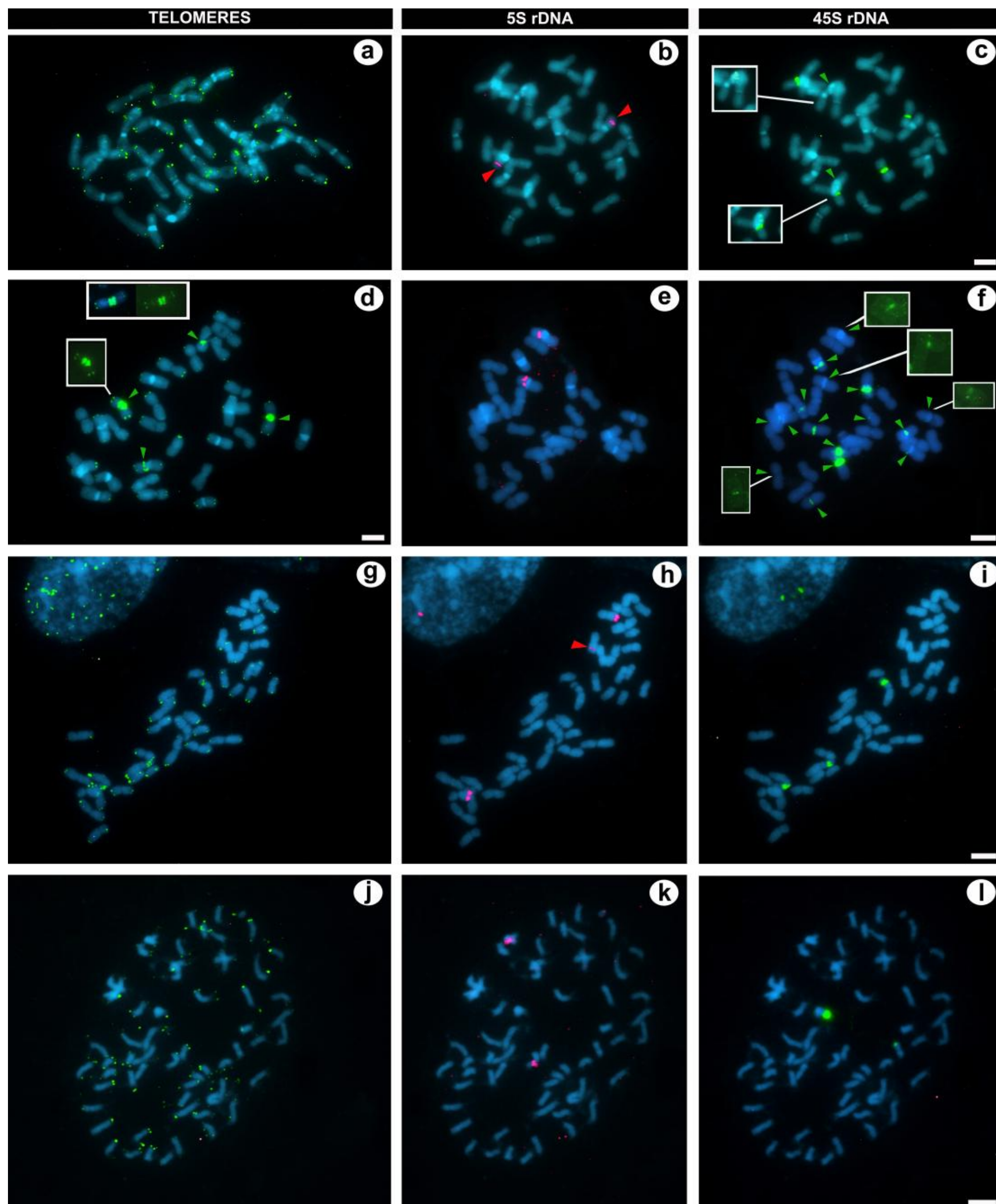


Fig. 3 Detection of telomeric signals, 5S and 45S rDNA sites in chromosomes of (a–c) *Spathiphyllum pygmaeum* (2*n* = 30), (d–f) *Spathiphyllum tenerum* (2*n* = 30), (g–i) *Cyrtosperma merkusii* (2*n* = 39), and (j–l) *Monstera deliciosa* (2*n* = 60) by FISH. Red arrowheads indicate the position of weak 5S rDNA sites in some cells, while green ones in (d) indicate the position of ITRs and in (f) of 45S rDNA signals. Insets in (d) display chromosome with telomeric probe without the overlapping with DAPI, and a chromosome from other cell (top) presenting similar telomeric distribution, and in (f) show chromosomes, without being overlapped with DAPI, with weak 45S rDNA signals treated with a differential brightness/contrast. Bars correspond to 5 µm, and are valid for plates in each row.

- **Discussion**

Modeling of chromosome number change in the Araceae family

The newly generated chromosome counts (Table 2) together with previously published numbers reveal an overall variation in the Araceae from $2n = 8$ (*Typhonium* spp.) to $2n = 160$ (*Lazarum* spp.). However, different from what one might expect from such numbers, polyploidy does not appear to have played a large role. Instead, our model-based maximum-likelihood inference of the likely direction in chromosome number change points to dysploidy as the predominant event in karyotype evolution in the family. This inference is now based on a family phylogeny with 163 species from all genera currently recognized, confirming an earlier study with just 112 species from 112 genera (Cusimano et al., 2012). A caveat in both analyses is that few chromosome counts are available for the outgroup families (Figs. 1 and S1) and that these families are phylogenetically far distant from the Araceae (which are the sister to a clade of all other Alismatales families), resulting in long genetic branches in the family phylogeny. To infer the most likely events, the ChromEvol approach (Mayrose et al., 2010) uses the frequencies of tip states (i.e., chromosome counts in the included species) together with branch lengths in gene trees (as a proxy for time). It is therefore not surprising that the long branches and few counts near the base of the Araceae phylogeny result in great uncertainty for the inferred events near the root. As an example, the ancestral chromosome number has no statistical support; it is $a = 16$ in our trees (Figs. 1 and S1) as in the phylogram of Cusimano et al. (2012, but $a = 18$ on their ultrametric tree). The subsequent evolutionary downward trend in chromosome numbers is strongly supported, however, going from $a = 16$ to 15 to 14 on the ultrametric tree (Fig. 1) and from $a = 16$ to 14 to 13 and back to 14 on the phylogram (Fig. S1).

Previously inferred basic chromosome numbers for the Araceae were $x = 7$ (Larsen, 1969; Marchant, 1973) or $x = 14$ (Petersen, 1993). These hypotheses were based on many fewer counts and a more limited understanding of phylogenetic relationships in the family compared to today. Especially important as regards chromosome number evolution in this family is the recognition that the five genera of Lemnoideae (in the past treated as Lemnaceae) are an early-divergent clade of the Araceae; all lemnoids have relatively high chromosome numbers ($2n = 20$ to $2n = 126$; Cao, 2013). The haploid numbers found in this and other early lineages of Araceae are high (based on $n = 13$, 14, 15, 20, 24, and 30; Figs. 1

and S1: tips), leading to the inference of $a = 16$ as a possible ancestral haploid number in the newly circumscribed Araceae (Cusimano et al., 2012; the present study). Nevertheless and as stressed above, the inferences near the root have no statistical support and might change which the inclusion of more outgroup chromosome numbers and more early Araceae lineages.

No evidence for polyploidy from the FISH data

We performed FISH in 14 Araceae species of which 11 belong to early lineages of the family and three to derived lineages. Although chromosome numbers are known for some 26% of the ca. 3790 species (Cusimano et al., 2012: Table S1), FISH studies were only begun recently, focusing on a relatively derived genus (Sousa et al., 2014). In the present study, we therefore sampled earlier-diverging lineages of Araceae, namely *Anthurium*, *Pothos*, *Stenospermatium*, *Spathiphyllum*, *Monstera*, *Scindapsus*, and *Rhaphidophora* (Figs. 1 and S1-S3). The FISH results for these genera showed a conserved number of 5S rDNA sites (one) but variable numbers of 45S rDNA sites (one to eight; see Table 2). Atypical numbers of rDNA signals (3 instead of 4) were observed in *Cyrtosperma merkusii* ($2n = 39$; Fig. 3i) and *Englerarum hypnosum* ($2n = 24$; Fig. S4e). The evolutionary event that led to the reduction of rDNA sites in these species, either loss of an entire chromosome or just of the 45S rDNA locus, remains unclear. Also unclear is the evolutionary significance of odd chromosome numbers (Fig. 3h-i), such as found here in *Cyrtosperma merkusii* and earlier in *Amorphophallus*, *Anthurium*, *Apoballis*, *Arisaema*, *Caladium*, *Cryptocoryne*, *Piptospatha*, *Schismatoglottis*, *Typhonium*, and *Xanthosoma* (Cusimano et al., 2012: Table S1; Sousa et al., 2014).

Our FISH work revealed no correlation between the number of rDNA sites and ploidy level. *Spathiphyllum* species with $2n = 30$ had three or eight 45S rDNA sites (*S. pygmaeum* and *S. tenerum*, Fig. 3c, f), while an *Anthurium* species with the same chromosome number (*A. leuconerum*, $2n = 30$) had two sites (Fig. 2o), and another pair of close relatives, both with $2n = 60$, had one or two 45S rDNA sites (*Monstera deliciosa* and *Scindapsus lucens*, Figs. 3l and S1c). Polyploids may have twice the rDNA sites as their parental species (additive polyploidy; see Adams et al., 2000; Ansari et al., 2008; Sousa et al., 2014), but we found no such case. Interestingly, multiple rDNA sites found in the *Spathiphyllum* were mainly located in the pericentric region close to or within heterochromatic DAPI-positive bands (Fig. 3c, f).

Pericentric regions are prone to the insertion of mobile elements (Mai et al., 2007), which can mediate the amplification of rDNA in a genome (Raskina et al., 2008: review).

Huge interstitial telomere repeats (ITRs)

Telomere motif repeats at both ends of each chromosomes were seen in all species studied here (Fig. 2a, d, j; Fig. 3a, g, j; Fig. S4a, d, f, h), but three species had additional interstitial telomere repeats (Figs. 2e, 3d, and S4g). Unexpectedly, we found no ITRs in the two *Ulearum* species with the largest chromosomes, while *Anthurium leuconerum* and *A. wendlingeri* (Figs. 2m and S4d, g), with medium-sized chromosomes, had ITRs in most or all chromosomes. These sites were located close to the centromere or in subterminal regions (Fig. 2m), and their number (12 and multiple signals) is the highest so far reported for any angiosperms. That they were discovered in *Anthurium* was unexpected because 80% of the 171 species of *Anthurium* that have had their chromosomes counted (out of 835 species in the genus) have counts of 2*n* = 30 (Cusimano et al., 2012: Table S1). This consistent chromosome number makes the discovery of ITRs, which are a sign of chromosome restructuring, surprising.

Interstitial telomeric sites are rare, but are known from *Vicia faba* (Schubert et al., 1995; Fuchs *et al*., 1995: Fig. 1), *Eleocharis subarticulata* (Da Silva et al., 2005), *Sideritis montana* (Raskina et al., 2008), and two species of *Typhonium* (Sousa et al., 2014). In *Vicia faba*, presence of ITRs was related to the existence of fusion-fission cycles, and in *Typhonium* to Robertsonian-fusion-like rearrangements. The latter mechanism differs from the former in involving the formation of a chromosome with a single centromere after a reciprocal translocation involving two acro- or telocentric chromosomes (Sousa et al., 2014). *Anthurium leuconerum* has one ITR per chromosome of a hybridization intensity similar to that of at the two chromosome ends. By contrast, *A. wendlingeri* has large ITR bands (Fig. S4g), indicative of massive repeat amplification. Such large ITR bands have so far only been reported from the gymnosperms *Cycas revoluta*, *Pinus elliottii* var. *elliottii, Pinus densiflora*, *Pinus taeda*, and *Pinus sylvestris* (Fuchs et al., 1995; Hizume et al., 1998; Schmidt et al., 2000; Shibata et al., 2005; Islam-Faridi et al., 2007), and in these species generally each chromosome displays more than one signal (up to 6). In *P. elliottii* var. *elliottii* and *P. densiflora*, some of the ITRs co-localize with positive DAPI bands, while the regular terminal telomere signals could not be detected or could be visualized only after differential brightness/contrast treatment

(Schmidt et al., 2000; Shibata et al., 2005; similarly in *P. taeda*, Fuchs et al., 1995). In the Araceae studied here, we also found co-localization of ITRs and positive DAPI bands in *Anthurium wendlingeri* and *Spathyphyllum tenerum* (Figs. 3d: inserts and S4g), suggestive of two chromosomes fused without being involved in a reciprocal translocation. Such an event would be incompatible with the telomeres' regular function in protecting chromosome ends from fusion (Schubert and Lysak, 2011).

Telomere lengths range from 2 to 5 kb in *Arabidopsis*, 2-40 kb in corn, 20-60 kb in tomato, >150 kb in tobacco (Lamb et al., 2012), and up to 20 kb in *Pinus* (Schmidt et al., 2000; Lamb et al., 2012). No estimates are available for any Araceae. The high number of interstitial telomere sites discovered in *Anthurium leuconerum* and *A. wendlingeri* (Figs. 2m and S4g) along with the signal brightness must indicate huge repeat-amplifications, so far unlinked to obvious karyotype changes. In *Spathyllum tenerum* (Fig. 3d), however, we could link the ITRs to Robertsonian fusion-like chromosome rearrangement, similar to the ones found in *Typhonium laoticum* (Sousa et al., 2014). Whatever their ultimate explanation, massive ITR bands as reported here suggest that nuclear genome assembly in the Araceae may be challenging. The importance of the FISH approach, especially multicolor FISH, as an aid in the *de novo* assembly of genomes of non-model plant species including Araceae is just beginning to be realized (Chamala et al., 2013: *Amborella*; Cao et al., 2013: Lemonoid Araceae).

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- # Appendices

Table S1 List of species used in this study with author names, herbarium vouchers, and GenBank accession numbers for all sequences. Herbarium acronyms follow the Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp).

Table S1.

Taxon name	Voucher (Herbarium)	Source	*trnL*	*trnL-F*	*trnK/matK*	*rbcL*
Chloranthaceae						
Hedyosmum orientale Merrill & Chun	T. Feild & Li 23 (PE)	Zhang and Renner, 2003	AY236749	AY236749	-	-
Alismataceae						
Alisma lanceolatum With.	(1) A. Jacobson pers. herb. (2) 2008/579 (BM)	(1) Jacobson and Hedren, 2007 (2) Schaefer et al., 2011	DQ354893 (1)	-	-	HM849753 (2)
Alisma orientale (Sam.) Juz.	A. Jacobson pers. herb.	Jacobson and Hedren, 2007	DQ354899	-	-	-
Caldesia grandis Samuelsson	L.-Y. Chen s.n. (Wuhan Botanical Garden)	Chen et al., 2012	-	-	JF781068	JF781043
Butomaceae						
Butomus umbellatus L.	(1) H. Solstad & R. Elven 145126 (O) (2) Li and Zhou unpublished (3) D.H. Les 499 (CONN)	(1) Lindqvist et al., 2006 (3) Les et al. 1997	DQ786413 (1)	DQ786413 (1)	AY952416 (2)	BUU80685 (3)
Hydrocharitaceae						
Hydrilla verticillata (L.f.) Royle	(1) Botanical Garden, Wuhan, China 06 (2) N. Tanaka 95125 (TI)	(1) Chen et al., 2010 (2) Tanaka et al., 1997	-	-	AB002571 (1)	AB004891 (2)
Hydrocharis dubia (Bl.) Backer	(1) Xingkaihu, Heilongjiang, China. 07, 2009 (2) N. Tanaka 95122 (TI)	(1) Chen et al., 2010 (2) Tanaka et al., 1997	-	-	AB002572 (1)	AB004892 (2)
Scheuchzeriaceae						
Scheuchzeria palustris L.	(1) N. Orderud 260398 (O) (2) D.H. Les s.n. (CONN)	(1) Lindqvist et al., 2006 (2) Les et al., 1993	DQ786414 (1)	DQ786414 (1)	-	SPU03728 (2)
Araceae						
Alocasia longiloba Miq.	(1) DCN 2669 Edinburgh (E) (2) P. Boyce AL193 (SAR) (3) J. Bogner 2645 (M)	Nauheimer et al., 2012a	JQ238667 (3)	JQ238752 (2)	JQ238839 (1)	-

Alocasia macrorrhizos (L.) Don	M. P. Medecilo 435 (De La Salle University, Dasmarinas Herbarium, DLSU-DH)	Nauheimer et al., 2012a	-	-	JQ238841	-
Anadendrum microstachyum (De Vries & Miq.) Backer & Alderw.	A. Hay 2022, 1982-4984 (K)	Tam et al., 2004	AY398559	AY398559	-	-
Anthurium leuconeurum Lem.	J. Bogner 3007 (M)	This study	KJ128248	KJ128248	-	-
Anthurium wendlingeri G.M.Barroso	J. Bogner 2684 (M)	This study	KJ128249	KJ128249	-	-
Anubias heterophylla Engl.	D. Barabé and A. Archambault 197 (MT)	Barabé et al., 2004	AY555168	AY555168	-	-
Asterostigma cryptostylum Bogner	E. G. Gonçalves 640 (UB)	Gonçalves et al., 2007	EF173565	EF173565	-	-
Chlorospatha longipoda (K. Krause) Madison	T. Croat et al. 82326 (MO)	This study	-	AF521872	-	-
Cryptocoryne fusca deWit	FL09-07 (Sarwak herbarium)	Lanying et al., Unpublished	-	-	JX258142	JX196340
Cryptocoryne lingua Becc. ex Engl.	M. W. Chase 10998 (K)	Cabrera et al., 2008	-	AM933329	AM920601	AM905779
Cyrtosperma cuspidispathum Alderw.	T. Croat 81515A (MO)	Rothwell et al., 2004	-	AY290836	-	-
Cyrtosperma merkusiii (Hassk.) Schott	J. Bogner 1954 (M)	This study	KJ128250	KJ128250	-	-
Gorgonidium vermicidum (Speg.) Bogner and Nicolson	Forzza 1965 (SPF)	Gonçalves et al., 2007	EF173582	EF173582	-	-
Gymnostachys anceps R. Br.	J. Bogner 2973 (M)	This study	KJ128251	KJ128251	-	-
Homalomena speariae Bogner & M.D. Moffler	E. Spear s.n. (M)	Nauheimer et al., 2012b	KC466578	-	KC466578	KC466589
Monstera deliciosa Liebm.	J. Bogner 2439 (M)	This study	KJ128252	KJ128252	-	-
Monstera deliciosa Liebm.	(1) D. Barabé 158 (MT) (2) PS3002MT01 (Herbarium of the Institute of Medicinal Plant Development, Beijing)	(1) Barabé et al., 2002 (2) Chen et al., 2010	AY054733 (1)	AY054733 (1)	-	GQ436772 (2)
Peltandra virginica Kunth	(1) J. Bogner 2119 (M) (2) D. Barabé 152 (MT) (3) M. Chase 11770 (K)	(1) Cusimano et al., 2012 (2) Barabé et al., 2002 (2)	AY054707 (2)	AY054707 (2)	EU886583 (1)	AM905815 (3)

		(3) Cabrera et al., 2008				
Philodendron oxycardium Schott	Y. Qiu 96053 (IU)	Cho and Palmer, 1999	-	-	-	AJ005623
Pinellia peltata Nimmo	T.S. Yi 08016 (KUN)	Li et al., 2012	JQ237232	JQ237232	-	JQ237202
Pothos repens (Lour.) Druce	J. Bogner 2284 (M)	This study	KJ128253	KJ128253	-	-
Rhaphidophora glauca (Wall.) Schott	C. Grey-Wilson & Phillips 64A, 1973-2244 (K)	Tam et al., 2004	AY398524	AY398524	-	-
Rhaphidophora pteropoda (Teijsm. & Binn.) Engl.	J. Bogner 2989 (M)	This study	KJ128254	KJ128254	-	-
Schismatoglottis calyptrata (Roxb.) Zoll. & Moritzi	D. Barabé & A. Archambault 194 (MT)	Barabé et al., 2004	AY555172	AY555172	-	-
Scindapsus lucens Bogner & P.C.Boyce	J. Bogner 2113 (M)	This study	KJ128255	KJ128255	-	-
Spathiphyllum pygmaeum Bogner	J. Bogner 3002 (M)	This study	KJ128256	KJ128256	-	-
Spathiphyllum tenerum Engl.	J. Bogner 2993 (M)	This study	KJ128257	KJ128257	-	-
Stenospermatium popayanense Schott	J. Bogner 463 (M)	This study	KJ128258	KJ128258	-	-
Stylochaeton bogneri Mayo	M. W. Chase 10685 (K)	Cabrera et al., 2008	-	AM933327	AM920598	AM905776
Typhonium circinnatum Hett. & J.Mood	W. Hetterscheid H.AR.248 (L, spirit coll.) = M. V. Silber 2 (M) from H.AR.	Cusimano et al., 2010	-	-	EU886551	-
Typhonium echinulatum Hett. & Sookchaloem	W. Hetterscheid H.AR.225 (L, spirit coll.) = M. V. Silber 6 (M)	Cusimano et al., 2010	-	-	EU886554	-
Ulearum donburnsii Croat & B.Feuerstein	J. Bogner 84834 (M)	This study	KJ128259	KJ128259	-	-
Ulearum sagittatum Engl.	J. Jangoux et al. INPA138864 (M)	This study	KJ128260	KJ128260	-	-
Urospatha decipiens Schott	J. Bogner 2866 (M)	This study	KJ128261	KJ128261	-	-

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Table S2 Information on the genera newly studied here. # species refers to the total number of species in a genus; # species *2n* known refers to the number of species with published chromosome counts; the percentage refers to these two numbers; 2n variation refers to the range of published diploid counts. Chromosome numbers in bold indicate the most representative 2*n* number(s). An asterisk marks a genus with many reports of B chromosomes.

Genera	**# species**	**# species 2*n* known**	**%**	***2n* variation**	**References**
*Anthurium**	905	171	19	20, 24, 26, 28, **30**, 31, 32, 34, 36, 40, 48, 49, 56, **60**, 66, 84, ca. 90, ca. 124	Cusimano et al. (2012); www.aroid.org/genera/130307 uberlist.pdf
Cyrtosperma	13	4	30	24, **26**	Cusimano et al. (2012); www.aroid.org/genera/130307 uberlist.pdf
Englerarum	1	1	100	24	Cusimano et al. (2012); http://www.aroid.org/aroid/
Monstera	ca. 40	5	12	24, 56, 58, **60**	Cusimano et al. (2012); Andrade and Mayo (1994)
Rhaphidophora	ca.100	8	8	26, 42, 54, 56, **60**, ca. 120	Boyce (2001); Cusimano et al. (2012)
Scindapsus	ca. 35	8	23	48, **56**, 58, **60**, 64	Bogner and Boyce (1994); Cusimano et al. (2012)
Spathiphyllum	49	9	18	18, **30**, 60	Cusimano et al. (2012); www.aroid.org/genera/130307 uberlist.pdf
Stenospermatium	50	4	8	28	Cusimano et al. (2012); www.aroid.org/genera/130307 uberlist.pdf
Stylochaeton	25	4	16	**28**, **56**	Cusimano et al. (2012); www.aroid.org/genera/130307 uberlist.pdf
Ulearum	2	2	100	14	Cusimano et al. (2012);

					http://www.aroid.org/aroid/
Pothos	57	4	5,7	**24**, 36, 60	Cusimano et al. (2012); IPCN; Boyce (2000) www.aroid.org/genera/130307 uberlist.pdf
Alocasia	78	23	29	24, 26, **28**, 40, 42, **56**, 68, 70, 84	Boyce (2008), www.aroid.org/genera/130307 uberlist.pdf

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International Aroid Society webpage (http://www.aroid.org/aroid/)

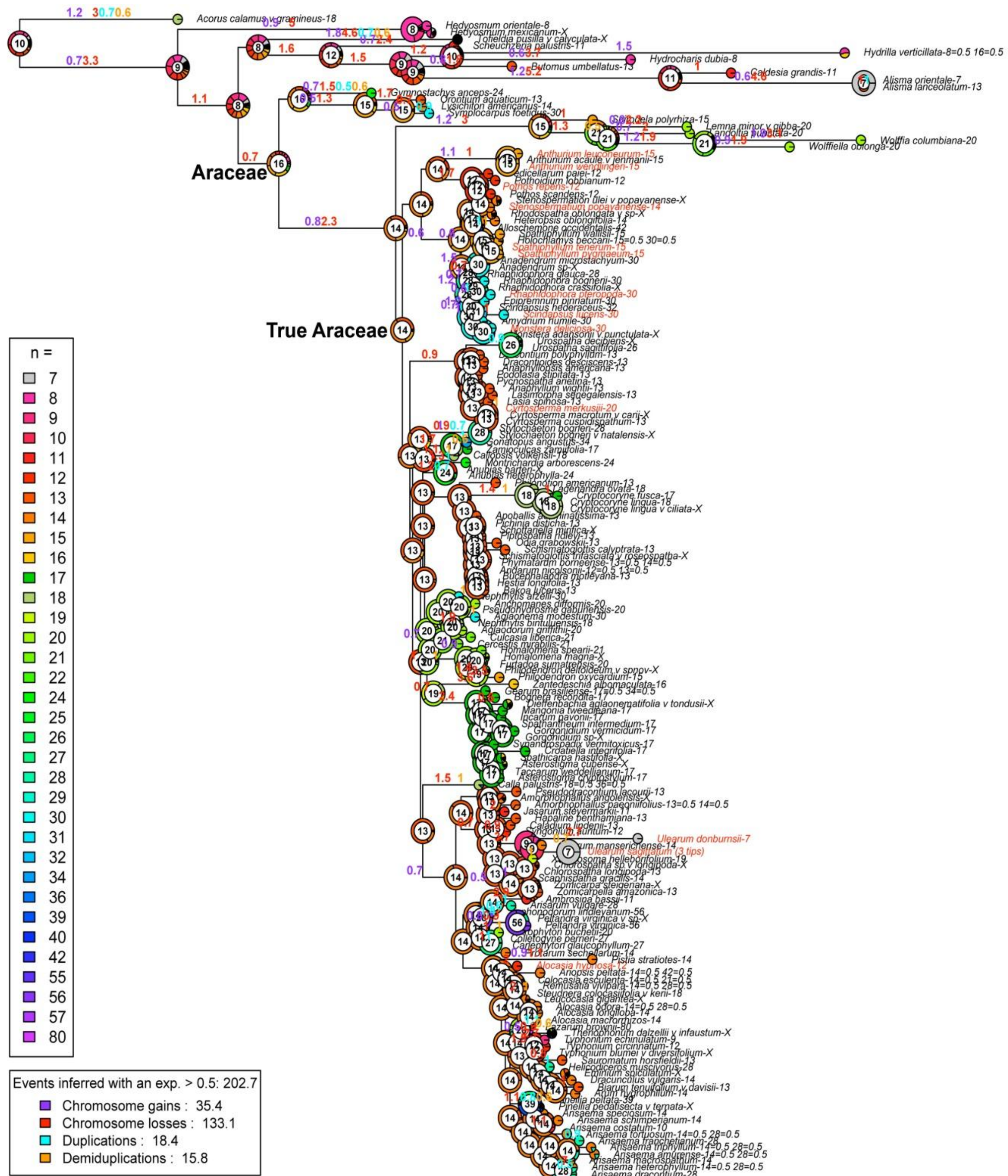


Fig. S1 Chromosome number reconstruction for Araceae on a phylogram, rooted on *Acorus calamus*. Pie charts represent the probabilities of inferred chromosome numbers, with the number inside pie having the highest probability. Numbers above branches are color-coded by event type (gains, losses, duplications, demiduplication) as shown in the rectangular inset and represent the frequency with which event type(s) with a probability >0.5 occurred along that branch. The color-coding of chromosome numbers is explained in the elongate inset on the left. Species investigated by FISH are labeled in red.

Fig. S2 Chromosome number reconstruction for Araceae on a phylogram tree rooted on *Acorus calamus*. Bootstrap supports are indicated at nodes and the inferred frequency of the four possible events (gains, losses, duplications, demiduplications) with a probability >0.5 are shown above branches. The color-coding of event types is explained in the inset. Species investigated by FISH are labeled in red.

Fig. S3 Chromosome number reconstruction for Araceae on an ultrametric tree rooted on *Acorus calamus*. Posterior probabilities are indicated at nodes and the inferred frequency of the four possible events (gains, losses, duplications, demiduplications) with a probability >0.5 are shown above branches. The color-coding of event types is explained in the inset. Species investigated by FISH are labeled in red.

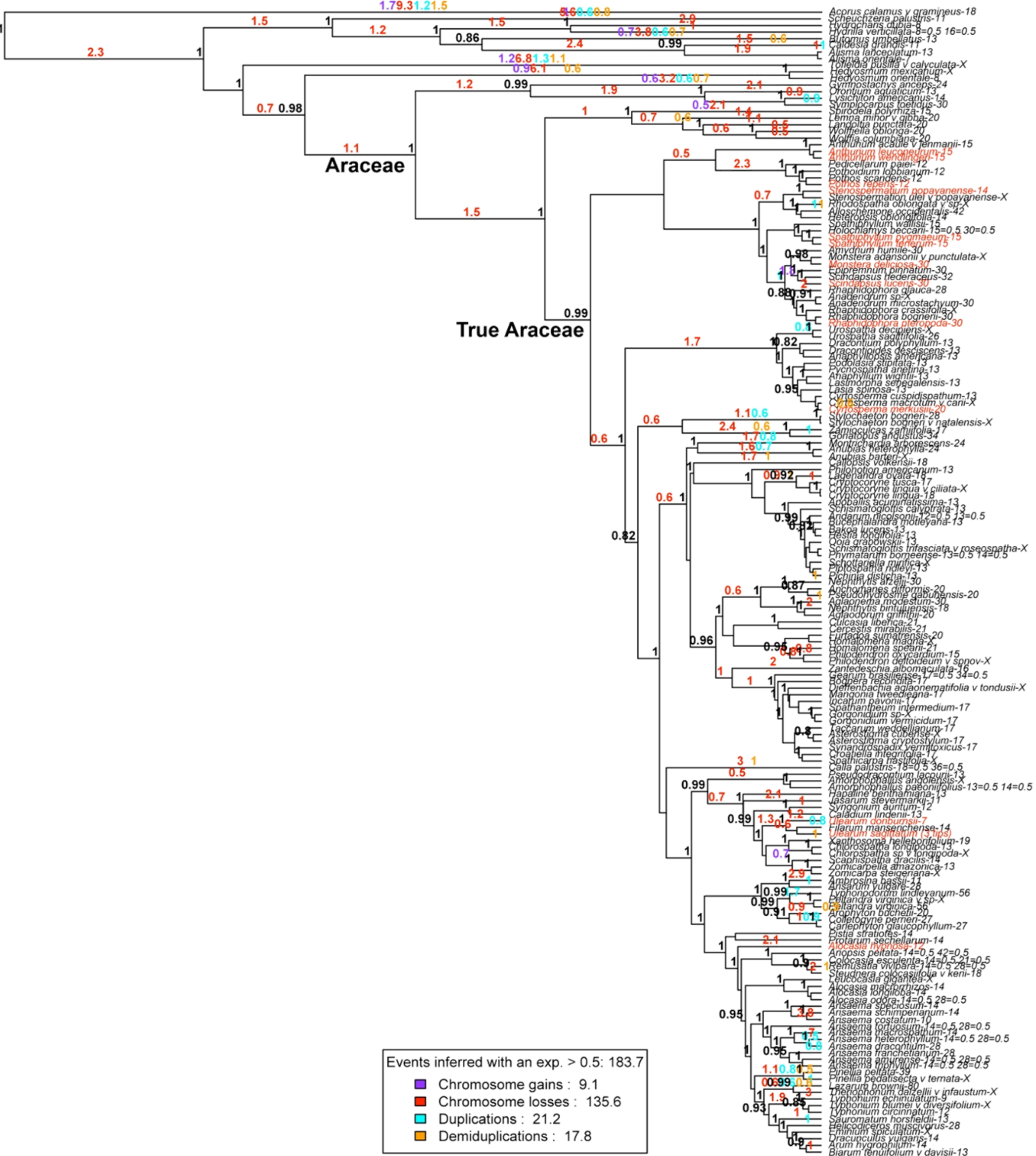
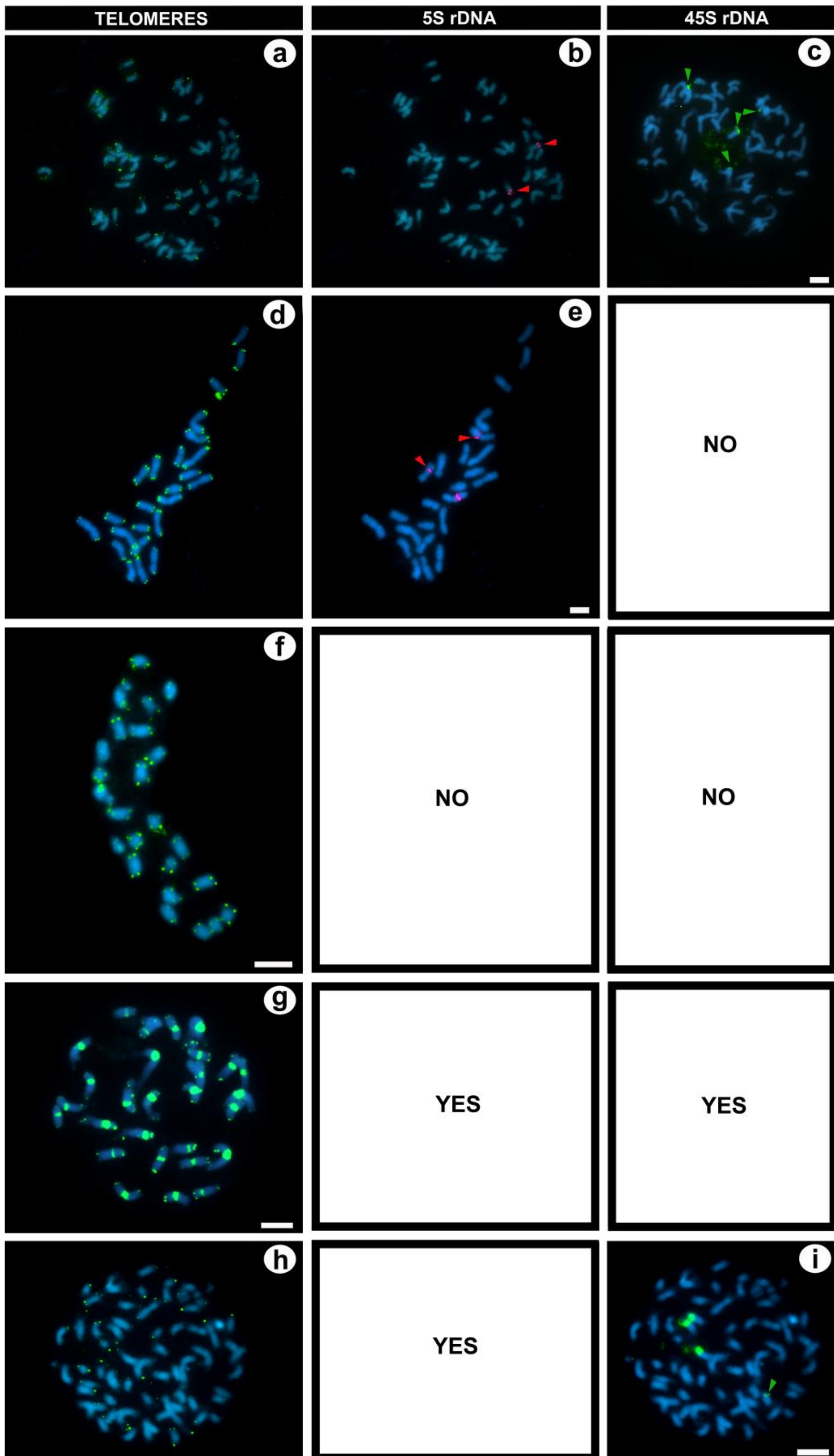


Fig. S4 (*facing page*) Detection of telomeric signals, 5S and 45S rDNA sites in chromosomes of (a–c) *Scindapsus lucens* (2*n* = 60); of telomeric signals and 5S rDNA sites in chromosomes of (d–e) *Englerarum hypnosum* (2*n* = 24); of only telomeric signals in chromosomes of (f) *Pothos repens* (2*n* = 24) and (g) *Anthurium wendlingeri* (2*n* = 30); and of telomeric signals and 45S rDNA sites in chromosomes of (H-I) *Rhaphidophora pteropoda* (2*n* = 60) by FISH. Red arrowheads indicate the position of weak 5S rDNA sites, while green ones in (c) and (i) indicate the position of weak 45S rDNA signals. Empty plates named by NO indicate that experiments using these probes were not made in these species while by YES means that they were performed but the experiment did not work or the results were unsatisfactory. Bars correspond to 5 μm, and are valid for plates in each row.


TELOMERES
5S rDNA
45S rDNA
a
b
c
d
e
NO
f
NO
NO
g
YES
YES
h
YES
i

Chapter 5

Molecular Cytogenetics (FISH, GISH) of *Coccinia grandis*: A ca. 3 myr-old species of Cucurbitaceae with the largest Y/autosome divergence in flowering plants.

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Molecular Cytogenetics (FISH, GISH) of *Coccinia grandis*: A ca. 3 myr-Old Species of Cucurbitaceae with the Largest Y/Autosome Divergence in Flowering Plants

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Key Words
5S and 45S rDNA · C-Banding · FISH · GISH · Sex chromosome · Telomeres

Abstract
The independent evolution of heteromorphic sex chromosomes in 19 species from 4 families of flowering plants permits studying X/Y divergence after the initial recombination suppression. Here, we document autosome/Y divergence in the tropical Cucurbitaceae *Coccinia grandis*, which is ca. 3 myr old. Karyotyping and C-value measurements show that the *C. grandis* Y chromosome has twice the size of any of the other chromosomes, with a male/female C-value difference of 0.094 pg or 10% of the total genome. FISH staining revealed 5S and 45S rDNA sites on autosomes but not on the Y chromosome, making it unlikely that rDNA contributed to the elongation of the Y chromosome; recent end-to-end fusion also seems unlikely given the lack of interstitial telomeric signals. GISH with different concentrations of female blocking DNA detected a possible pseudo-autosomal region on the Y chromosome, and C-banding suggests that the entire Y chromosome in *C. grandis* is heterochromatic. During meiosis, there is an end-to-end connection between the X and the Y chromosome, but the X does not otherwise differ from the remaining chromosomes. These findings and a review of plants with heteromorphic sex chromosomes reveal no relationship between species age and degree of sex chromosome dimorphism. Its relatively small genome size (0.943 pg/2C in males), large Y chromosome, and phylogenetic proximity to the fully sequenced *Cucumis sativus* make *C. grandis* a promising model to study sex chromosome evolution.

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Sex chromosomes in land plants are known from 48 species in 20 families of liverworts, gymnosperms, and flowering plants, where they evolved independently and over widely different time spans [Ming et al., 2011]. Indeed, the sex chromosomes of liverworts differ so fundamentally from those of vascular plants in functioning during the haploid phase of the life cycle, that they might better be considered a third chromosomal sex-determining system, besides X/Y and W/Z systems [Bachtrog et al., 2011]. These independent origins offer the opportunity to compare incipient sex chromosomes, such as those of *Papaya* and *Fragaria*, which are just 0.5–2.2 myr old [Liu et al., 2004; Spigler et al., 2008, 2010; Yu et al., 2008], with older ones, such as those of *Silene* or *Rumex*, which are thought to be over 10 myr old [Moore et al., 2003; Navajas-Pérez et al., 2005; but see Discussion section]. So far,

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heteromorphic sex chromosomes are reliably known from 19 species of Cannabaceae, Caryophyllaceae, Cucurbitaceae, and Polygonaceae [Ming et al., 2011]. About half of the 19 species have been studied with molecular-genetic tools (e.g. Sakamoto et al. [2000]: *Cannabis* (Cannabaceae); Karlov et al. [2003], Divashuk et al. [2011], Grabowska-Joachimiak et al. [2011]: *Humulus* (Cannabaceae); Ruiz Rejón et al. [1994], Shibata et al. [1999, 2000], Mariotti et al. [2006, 2009], Cuñado et al. [2007]: *Rumex* (Polygonaceae); Uchida et al. [2002], Lengerova et al. [2004], Hobza et al. [2006], Cermak et al. [2008], Kejnovsky et al. [2009]: *Silene* (Caryophyllaceae)).

Conspicuously neglected among the plants with heteromorphic sex chromosomes is the Cucurbitaceae *Coccinia grandis*. Classic cytogenetic work established that the Y chromosome in this species is much larger than the other chromosomes [Kumar and Deodikar, 1940; Bhaduri and Bose, 1947; Chakravorti, 1948; Kumar and Vishveshwaraiah, 1952], and experimental work in the 1970s confirmed the sex-determining role of the single Y chromosome [Roy and Roy, 1971]. In spite of the growing interest in plant sex chromosomes [Ming et al., 2011], modern cytogenetic methods have not been applied to *C. grandis* nor has the size of its genome been determined. *C. grandis* belongs to a small genus (25 species) that is phylogenetically close to *Cucumis*, the genus containing cucumber and melon [Schaefer and Renner, 2011]. A dated molecular phylogeny for 24 *Coccinia* species including *C. grandis* is available [Holstein and Renner, 2011].

Here, we characterize the karyotype of male and female *C. grandis* using mitotic and meiotic cell preparations, flow cytometry, FISH with telomere and 5S and 45S rDNA probes, and GISH to evaluate differences between the sexes. We also review XY chromosome size differences in land plants (including the haploid-dominant bryophytes), relating the differences to species ages inferred from molecular-clock studies. The questions we wanted to answer were (i) if rDNA or end-to-end fusions likely have contributed to the elongation of the Y chromosome in *C. grandis* and (ii) if there is a relationship between the age of vascular plant sex chromosomes and the extent of X/Y or Y/autosome morphological divergence.

Materials and Methods

Plant Material

C. grandis (L.) Voigt (including the illegitimate name *C. indica* Wight & Arn.) ranges from tropical Africa to subtropical and tropical Asia and is an invasive weed on Hawaii, other Pacific islands, and in tropical Australia. It belongs to *Coccinia*, a genus of 25 species in sub-Saharan Africa, all of them dioecious climbers. A recent revision of the genus has clarified the boundaries among the species [Holstein, 2012], and a dated molecular phylogeny that includes all but one of the species indicates that the entire genus evolved over just 7 myr [Holstein and Renner, 2011].

Seeds for this study were collected in spring 2011 on the campus of Kakatiya University in Vidyaranyapura, located in the northern part of Bangalore, state of Warangal, India. In Munich, they were germinated on moist filter paper and then transferred to plastic pots with standard potting soil. Female and male plants were identified by chromosome preparations, and later verified by checking if their flowers were male or female. Plants are still in cultivation in the greenhouses of the Botanical Garden Munich, and a voucher has been deposited in the herbarium of Munich (Sousa and Silber 1 and 2).

Flow Cytometric Genome Size Measurement

Nuclei were isolated from young leaves of 1 male and 1 female *C. grandis*, the sex of which was known since the plants had flowered. Measurements were made on 2 leaves per sex, with each measurement repeated 6 times on 2 different days. Roughly 50 mm^2 of leaf tissue were co-chopped with equal amounts of young leaf tissue of *Glycine max*, cv. Cina 5202 'Voran' (IPK gene bank accession number SOJA 392; 2C = 2.23 pg; Borchert et al. [2007]) as an internal reference standard using a razor blade in a Petri dish containing 0.7 ml of nuclei isolation buffer [Galbraith et al., 1983] supplemented with 1% polyvinylpyrrolidone 25, 0.1% Tween 20®, DNase-free RNase (50 μg/ml) and propidium iodide (50 μg/ml). The nuclei suspension was filtered through a 35-μm-mesh cell strainer cap into a 5-ml polystyrene falcon tube. After at least 15 min of incubation, DNA content measurement was performed on the FACStar^{PLUS} cell sorter (BD Biosciences) equipped with an argon ion laser INNOVA 90C (Coherent). Approximately 10,000 particles per sample were analyzed, and fluorescence intensities of nuclei were measured using the software CELL Quest ver. 3.3 (BD Biosciences). The absolute DNA amounts were calculated based on the values of the G1 peak means.

Chromosome Preparation

Mitotic metaphase chromosomes were prepared from root tips pre-treated in 2 mM 8-hydroxyquinoline for 20 h at 4°C, fixed in freshly prepared 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and kept at –20°C. Fixed root tips were washed 3 times for 5 min in distilled water, digested with 1% cellulase (w/v; Onozuka RS, Serva), 0.4% pectolyase (w/v; Sigma), 0.4% cytohelicase (w/v; Sigma) in citric buffer, pH 4.8 for 30 min at 37°C, dissected in a drop of 45% acetic acid and squashed. Coverslips were removed after freezing in dry ice and preparations were air-dried at room temperature. The quality of spreads was checked microscopically using phase-contrast, and only preparations with at least 10 well-spread metaphases were used for FISH/GISH.

Meiotic preparations were made from anthers of young buds. Anthers were fixed in 3:1 (v/v) ethanol/glacial acetic acid at room temperature overnight and stored at –20°C. Fixed anthers were quickly washed in distilled water, dissected in a drop of 45% acetic acid and squashed. Coverslips were removed after freezing, air-dried at room temperature, and the best slides were stained with DAPI (2 μg/ml). After taking pictures, slides were destained

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in 3:1 (v/v) ethanol/glacial acetic acid at room temperature for 30 min, kept overnight at 10°C in 100% ethanol, air-dried and kept at room temperature until they were used for C-banding.

DNA Probes

The heterologous ribosomal DNA sequences used as FISH probes were the 18S-5.8S-25S rDNA repeat unit of *Arabidopsis thaliana* in the pBSK+ plasmid, labeled with digoxigenin-11-dUTP (Roche) by nick translation, and a 349-bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris* cloned into pBSK+ [Schmidt et al., 1994], labeled with biotin-16-dUTP (Roche) by PCR. An *Arabidopsis*-like telomeric probe was amplified by PCR according to Ijdo et al. [1991] using the oligomer primers $(5'\text{-TTTAGGG-}3')_5$ and $(5'\text{-CCCTAAA-}3')_5$ and labeled with digoxigenin-11-dUTP by nick translation.

For GISH, genomic DNA from *C. grandis* male and female plants was isolated using the DNeasy Plant Maxi Kit (QIAGEN). Genomic DNA (1 μg) was autoclaved for 2 min to a fragment size range of 200–400 bp and labeled with digoxigenin-11-dUTP or biotin-16-dUTP (Roche) by nick translation. Blocking DNA was obtained by autoclaving total genomic DNA for 5 min, yielding fragments of approximately 100–200 bp. In GISH experiments, the probe/block ratio was 1:47, 1:70 and 1:100. Digoxigenin-labeled probes were detected with anti-digoxigenin conjugated with FITC (Roche) and biotin-labeled probes with ExtrAvidin conjugated with Cy3 (Sigma).

FISH

FISH was carried out using the method of Schwarzacher and Heslop-Harrison [2000] with minor modifications. Slides were pre-treated with 100 μg/ml of RNase A in 2× SSC buffer for 1 h at 37°C and washed 3 times for 5 min in 2× SSC. They were then treated with 10 μg/ml Pepsin (Sigma) in 0.01 N HCl for 20 min at 37°C, washed twice for 5 min in 2× SSC, post-fixed in 4% formaldehyde solution (Roth) for 5 min at room temperature, washed again 3 times for 5 min in 2× SSC, dehydrated for 5 min in a 70 and 100% ethanol series and air-dried for at least 1 h at room temperature. Hybridization mixtures consisted of 50% formamide (w/v), 2× SSC, 10% dextran sulfate (w/v) and 70–200 ng of labeled probe. The hybridization mix was denatured at 75°C for 10 min and immediately cooled on ice for 10 min; 10–15 μl of the mix was then added to each slide and covered with a glass coverslip. For hybridization, the chromosomes, together with the hybridization mixture, were denatured for 5 min at 75°C. Hybridization was carried out in a humid chamber at 37°C for 20 h. After hybridization, the slides were washed 3 times for 5 min in 2× SSC at 42°C, 5 min in 2× SSC at room temperature and 5 min in 2× SSC/0.1% (v/v) Tween 20 at room temperature. For digoxigenin and biotin detection, slides were incubated in blocking buffer (2% BSA in 2× SSC) in a humid chamber for 30 min at 37°C, followed by incubation with anti-DIG-FITC conjugate (Roche) and streptavidin-Cy3 conjugate (Sigma) at 37°C for 1 h. Excess of antibody was removed by washing the slides twice for 7 min in 2× SSC and for 7 min in 2× SSC/0.1% (v/v) Tween 20 at 42°C. The chromosomes were counterstained with DAPI (2 μg/ml) and mounted in Vectashield (Vector).

GISH

The GISH procedure resembled the FISH procedure except that blocking DNA was added to the hybridization mixture. The latter thus consisted of 50% formamide (w/v), 2× SSC, 10% dextran sulfate (w/v), 83 ng of digoxigenin-labeled *C. grandis* male DNA probe, and 3,500–8,500 ng of non-labeled genomic DNA of a *C. grandis* female. To achieve a 1:47, 1:70 or 1:100 ratio between probe and blocking DNA we used *C. grandis* female DNA at concentrations of 3,928, 6,017 and 8,300 ng.

C-Banding

C-banding was performed according to Schwarzacher et al. [1980] with minor modifications. Slides were left for 3 d at room temperature and then incubated in 45% acetic acid at 60°C for 10 min, washed for 1 min in running tap water, dried using an air pump, and incubated in barium hydroxide (Roth) at room temperature for 10 min. The crystals of barium hydroxide were removed by briefly washing the slides in running tap water, followed by a rinse in 45% acetic acid, another 2 min in running tap water and a final rinse in distilled water. The slides were dried using an air pump, and incubated in 2× SSC at 60°C for 1 h 20 min. After the incubation, the slides were washed in distilled water, dried, counterstained with DAPI (2 μg/ml), and mounted in Vectashield (Vector).

Image Analysis

Images were taken with a Leica DMR microscope equipped with a KAPPA-CCD camera and the KAPPA software. They were optimized for best contrast and brightness using Adobe Photoshop CS3 version 10.0.

Karyotype Analysis

Chromosomes and positions of rDNA sites were measured using Adobe Photoshop CS3, and idiograms were constructed based on the analysis of 4 well-spread metaphases, with chromosomes ordered from the largest to the shortest pair, except for the Y chromosome. The X chromosome was assumed to be the smallest chromosome not pairing with an equal-sized autosome; no specific X probes are so far known for *C. grandis*. The chromosome arm ratio (AR, defined as length of the long arm/length of the short arm) was used to classify chromosomes as metacentric (AR = 1–1.4), submetacentric (AR = 1.5–2.9), or acrocentric (AR ≥ 3.0) following Guerra [1986].

Review of X/Y or Y/Autosome Size Differences in Land Plants

Vascular plants with heteromorphic sex chromosomes were tabulated based on Ming et al. [2011] and the most recent available data on their karyotypes, chromosome lengths, and male/female C-value differences were compiled from the literature. Divergence times for the relevant species inferred with molecular clocks were compiled from phylogenetic studies.

Results

Karyotype, Idiogram, Meiosis and FISH Results

C. grandis females have a karyotype of 2n = 22 + XX and males have 2n = 22 + XY. As the unpaired large chromosome correlates with maleness in the phenotype, *C. grandis* has heteromorphic sex chromosomes. On an agarose gel (online suppl. fig. 1, for all online suppl. materi-

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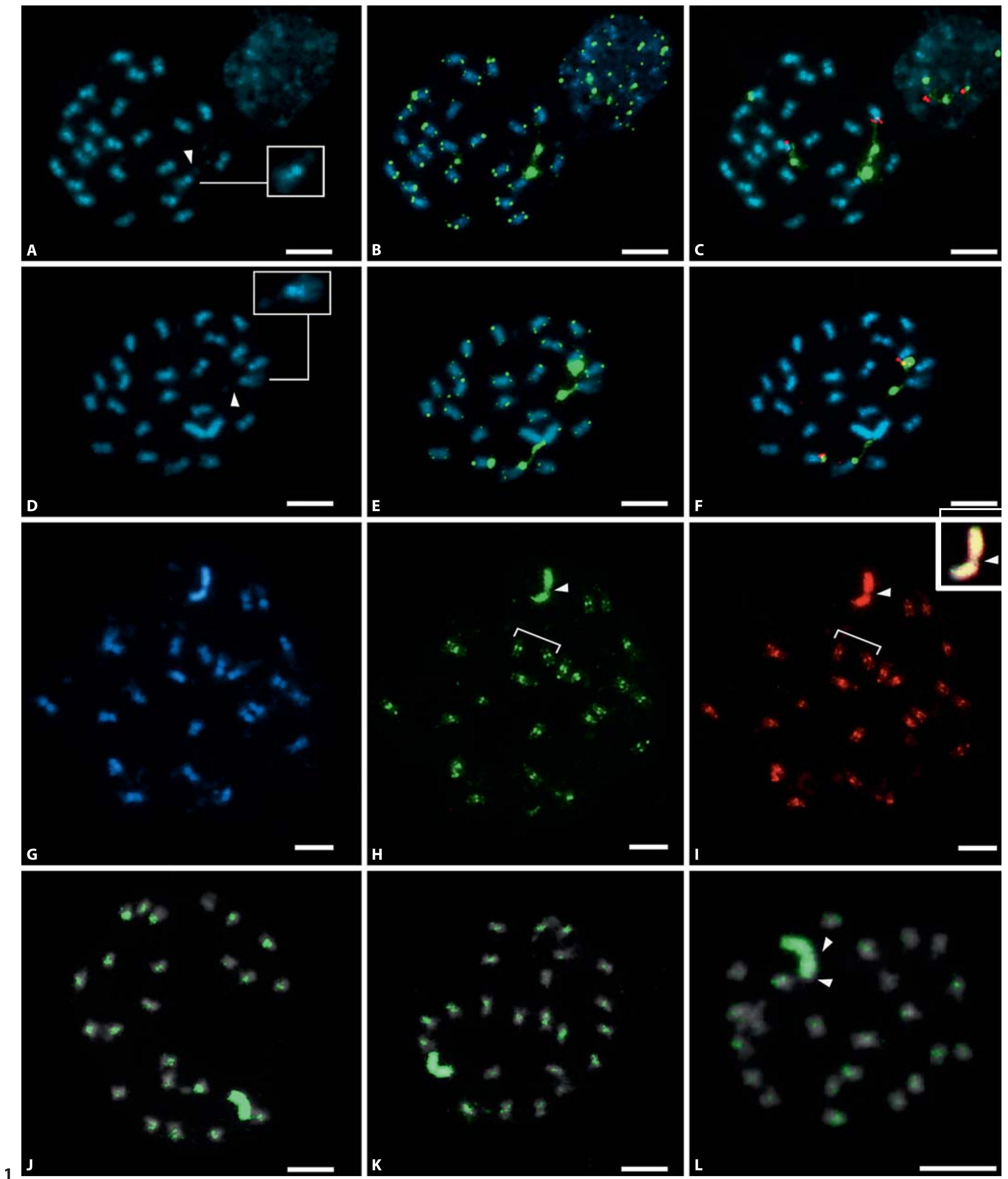

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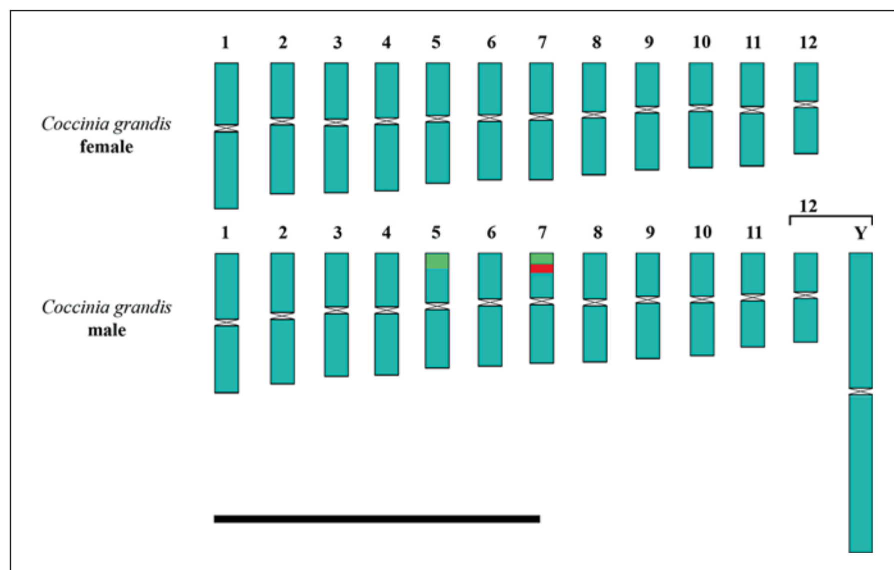

Fig. 2. Idiograms of the haploid chromosome complement of *C. grandis*, including 5S (red) and 45S (green) rDNA sites (although only shown in the male, they are equally distributed in the female). Chromosome pairs were put together by similarity. The X chromosome is shown beside the Y chromosome in the male. Telomere sites were detected in all chromosome ends and are not represented in this idiogram. Bar corresponds to 5 μm.

als, see www.karger.com/doi/10.1159/000345370), autoclaved *C. grandis* female DNA was more stable than male DNA.

Two 45S rDNA sites and one 5S rDNA site were detected in both male and female individuals. The two 45S rDNA sites were always located at the terminal regions of the chromosomes, and the 5S rDNA site was adjacent to one 45S rDNA site (fig. 1C, F). Secondary constrictions were observed in at least 1 chromosome per karyotype in both sexes (see arrowheads in fig. 1A, D and their insets). The *Arabidopsis*-like telomeric probe revealed telomere sequences at the ends of all chromosomes in both females and males (fig. 1B, E), but no interstitial telomere sites.

Fig. 1. FISH (**A–F**) and GISH (**G–L**) on mitotic metaphase chromosomes of *C. grandis*. **A**, **D** DAPI stained chromosomes (2n = 24) with 24 homomorphic chromosomes in a female plant, and 23 homomorphic chromosomes and a large heteromorphic Y chromosome in a male plant, respectively. **Insets** show magnified chromosomes with arrowheads marking satellites. **B**, **E** Distribution of telomeric sequences (small green dots located at the end of the chromosomes) and 45S rDNA (4 strong green signals). **C–F** Bicolor FISH with 45S rDNA (green) and 5S rDNA probe (red). DAPI male metaphase (**G**), and GISH using male genomic probe (**H**) and female genomic probe (**I**). Arrowheads in **H** and **I** show the Y centromere region; the **Inset** (**I**) shows an enlarged Y chromosome with its centromeric region not strongly labeled by either genomic probe. **J–L** GISH using 47×, 70×, and 100× excess of female blocking DNA, respectively. Arrowheads in **L** show small hybridization gaps. Scale bars correspond to 5 μm.

Figure 2 shows idiograms of *C. grandis* male and female individuals. rDNA sites are presented in figure 2 only in males; females had the same numbers and positions of rDNA.

In meiosis, 12 bivalents could be seen in late prophase I (diakinesis) and in the metaphase plate (fig. 3). Clear end-to-end connections between the X and the Y chromosome were observed (fig. 3A, C, E; as also reported by Bhaduri and Bose [1947]).

GISH and C-Banding Results

GISH experiments were performed with males, using male and female genomic probes. Figure 1H shows that the male genomic probe labeled the (peri-)centromeric and some subterminal regions plus the complete Y chromosome. When the same metaphase preparation was hybridized with the female genomic probe (fig. 1I), the centromeric regions and the Y chromosome again were intensely labeled. The overlap of male and female probes (fig. 1I, inset using DAPI in gray) on the Y chromosome shows that the centromeric region was not well-labeled in comparison to the other chromosomes (arrowheads fig. 1H, I), suggesting that the centromere sequences of the autosomes/X chromosome and the Y chromosome differ in DNA composition. In a few chromosomes, including the Y chromosome, the subterminal regions were predominantly labeled with male genomic probe (these chromosomes are marked by brackets in fig. 1H, I), indicating that subterminal repetitive sequences may have accumulated on the Y chromosome.

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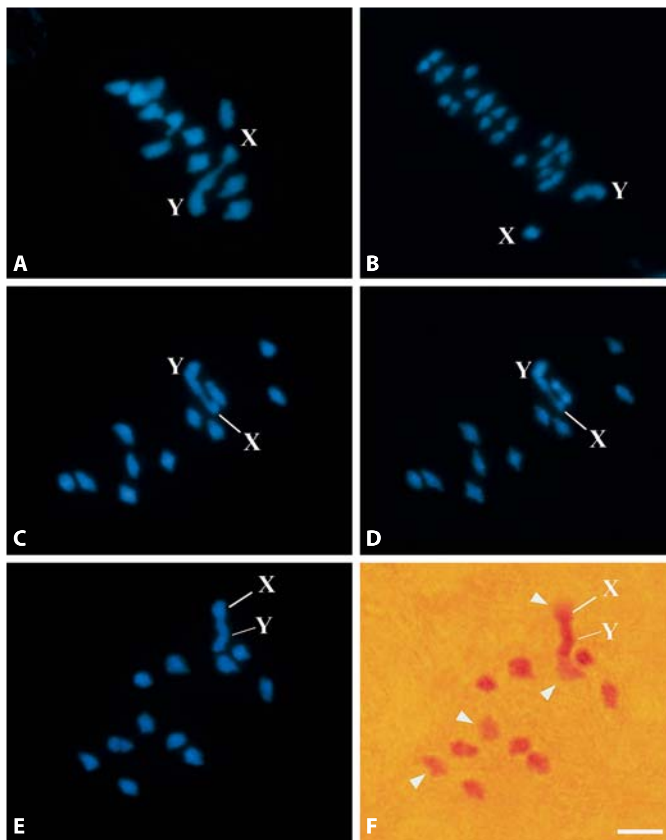

Fig. 3. Meiosis I in a *C. grandis* male, with the position of the sex chromosomes (X/Y) indicated in all cells. **A** Twelve bivalents in metaphase I moving together along the metaphase plate. **B** Early anaphase I with the migration of bivalents and X/Y chromosomes to opposite poles of the cell. **C**, **E** Cells stained with DAPI before C-banding (**D**, **F**). **D** Bivalents stained with DAPI showing no detectable morphological distinction between the X and autosomes. **F** Cell stained with Giemsa showing weakly stained bivalents (arrowheads) including the terminal region of X chromosome. Bar corresponds to 5 μm.

When male genomic probes were used with female blocking DNA in different concentrations, the intensity of the hybridization signals in the centromeric region of the chromosomes decreased or disappeared entirely with increasing concentration of female DNA. With 47× excess of blocking DNA, the Y chromosome was well-labeled as were most of the chromosomes (fig. 1J); with 70× excess of blocking DNA, the Y chromosome still was well-labeled, but a few chromosomes exhibited no or weak hybridization signals (fig. 1K); with 100× excess of blocking DNA, the Y chromosome started to present hybridization gaps not labeled by the male genomic probe, and 1 terminal region did not show any detectable hybridization signal (fig. 1L, arrowheads). The reduction of the signal strength presumably is associated with similar repetitive sequences shared by male and female *C. grandis*.

C-banded heterochromatic regions in females were mostly concentrated in centromeric/pericentromeric regions (fig. 4B, C) while in males (fig. 4E, F) they were diffusely pericentromeric/subterminal. The Y chromosome showed the same DAPI intensity before and after C-banding, again suggesting that the Y chromosome in *C. grandis* is heterochromatic. Using Giemsa staining (fig. 4C, F), female pre-metaphase chromosomes were all more or less well-stained while male pre-metaphases showed only few chromosomes, including the Y chromosome, with strong Giemsa-labeling.

In meiotic cells stained with DAPI, bivalents in metaphase I displayed few differences before and after C-banding (see fig. 3C, D). The terminal region of the X chromosome, but not the Y chromosome, was DAPI-positive, implying that the pseudoautosomal region is mainly euchromatic (fig. 3D). The autosomes were more intensely stained in the internal region of each bivalent, and no detectable morphological distinction could be observed between the X and the autosomes (fig. 3D). With Giemsa-staining (fig. 3F), some bivalents in late prophase I exhibited less labeling than others (see arrowheads) after C-banding, and the free terminal region of the X chromosome was less strongly labeled than its other end, connected to the Y chromosome.

Chromosome Measurements, C-Values and Comparison with Other Vascular Plant Sex Chromosomes

Chromosome lengths in the female varied from 1.35 to 2.26 μm and in the male from 1.33 to 4.71 μm. The largest autosome in males was 2.28 μm long, meaning that the Y chromosome, with 4.71 μm, is around twice as long as the largest chromosome. On the basis of their centromere position, all *C. grandis* chromosomes have an AR index of 1–1.4, making them metacentric (see table 1). Based on measurements on nuclei isolated from young leaf tissue, female individuals have a C-value of 0.849 pg/2C and male individuals of 0.943 pg/2C (table 2).

Table 3 summarizes data on X and Y chromosome lengths, C-values, and inferred ages for vascular plant species with heteromorphic sex chromosomes. Species with sex chromosomes are usually characterized by ARs (p/q) and relative, not absolute lengths because length to some extent depends on the preparation protocol and environmental factors. The data available so far reveal no relation-

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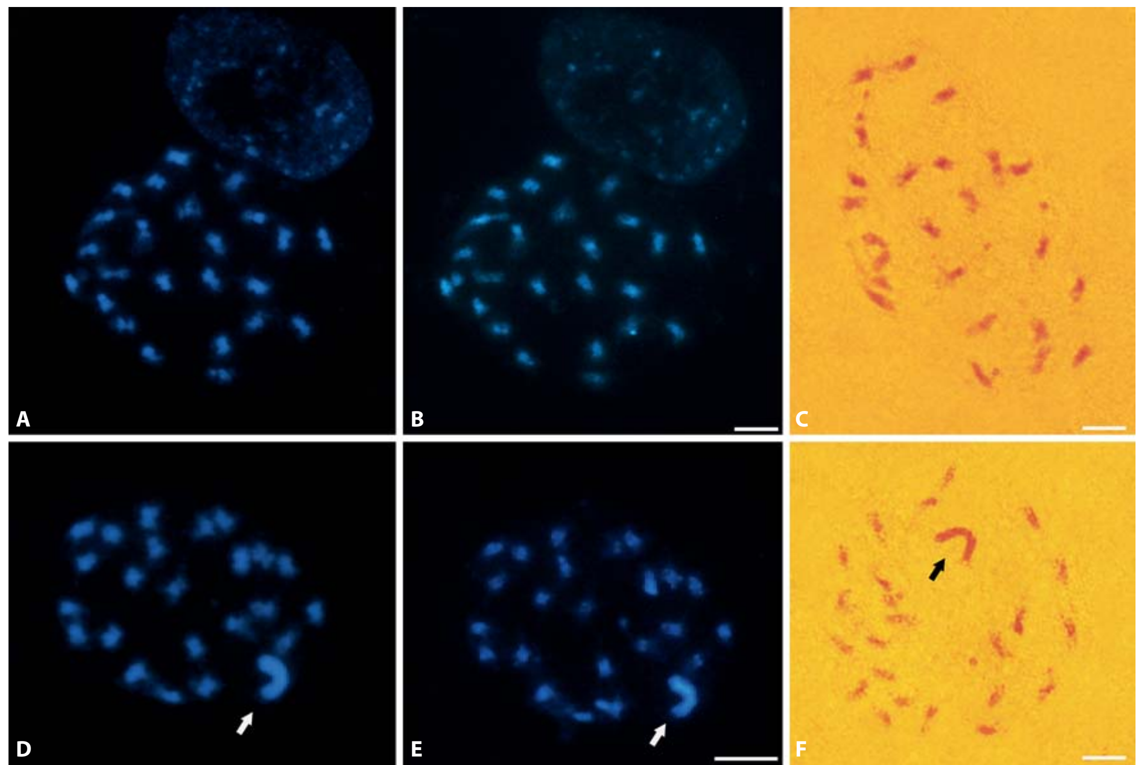

Fig. 4. C-banding in a *C. grandis* female (**A–C**) and male (**D–F**). **A**, **D** Metaphases stained with DAPI before C-banding. Chromosomes are stained along their entire length. **B**, **E** Metaphases showing centromeric and subterminal DAPI-positive regions, hence heterochromatic. The Y chromosome in **D** and **E** shows similar DAPI intensity before and after C-banding. **C**, **F** Pre-metaphases stained with Giemsa after C-banding. The heterochromatin is well-distributed on all chromosomes in **C** while it is concentrated on the Y chromosome in **F**. Arrows in **D–F** indicate the Y chromosome. Bar in **B** valid for **A**, and in **E** valid for **D**. Bars correspond to 5 μm.

Table 1. Morphology of *C. grandis* male and female chromosomes

Chromosome pairs	Chromosome size, μm		AR		Chromosome type	
	male	female	male	female	male	female
1	2.28 ± 0.25	2.26 ± 0.06	1.13	1.21	m	m
2	2.01 ± 0.14	2.03 ± 0.13	1.12	1.24	m	m
3	1.87 ± 0.14	2.01 ± 0.06	1.17	1.22	m	m
4	1.87 ± 0.14	1.96 ± 0.05	1.15	1.19	m	m
5[a]	1.75 ± 0.21	1.85 ± 0.03	1.19	1.16	m	m
6	1.73 ± 0.11	1.78 ± 0.02	1.28	1.14	m	m
7[b]	1.66 ± 0.14	1.75 ± 0.04	1.29	1.23	m	m
8	1.66 ± 0.14	1.69 ± 0.05	1.23	1.14	m	m
9	1.60 ± 0.06	1.65 ± 0.04	1.28	1.36	m	m
10	1.56 ± 0.03	1.60 ± 0.06	1.22	1.32	m	m
11	1.44 ± 0.04	1.58 ± 0.08	1.14	1.26	m	m
12X[c]	1.33 ± 0.05	1.35 ± 0.12	1.09	1.21	m	m
12Y	4.71 ± 0.34		1.18		m	

[a] Chromosome pairs with only 45S. [b] Chromosome pairs with 45S and 5S rDNA. [c] Likely X chromosome/X chromosome pair. m = Metacentric. The length of satellites is not included in the chromosome length.

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Table 2. Flow cytometric measurements for male and female *C. grandis*

	Leaves	Samples	DNA content, pg/2C	SD
Male	2	12	0.943	0.005
Female	2	12	0.849	0.005

Table 3. Chromosome numbers, lengths, 2C-values, and inferred age of sex chromosomes in vascular plant species with heteromorphic sex chromosomes

Species	Chromosome number, 2n	Chromosomal sex determination	X chromosome length μm	Y chromosome length μm	DNA content (2C) pg	Age of species or clade, myr	References (studies with age estimates)
Podocarpus macrophyllus	37, 38	X_1X_2/Y	unknown	9.1	unknown	unknown	Hizume et al. [1988]
Coccinia grandis	24	XX/XY	indistinguishable from autosomes	4.71 (10% of male genome weight)	M = 0.943 F = 0.849	3–6	Holstein and Renner [2011]; Holstein [2012]
Humulus japonicus	16, 17	XX/XY_1Y_2	3.11	Y_1 = 2.98 Y_2 = 2.75	M = 3.522	unknown	Grabowska-Joachimiak et al. [2006]
Humulus lupulus	20	XX/XY	2.39[a]	1.63	M = 5.523	unknown	Grabowska-Joachimiak et al. [2006]
Rumex acetosa	14, 15	XX/XY_1Y_2	3% of female genome weight	Y_1 = 7.5 Y_2 = 6.9 (20% of male genome weight)	M = 7.498 F = 7	15–16	Kurita and Kuroki [1970]; Błocka-Wandas et al. [2007]
Silene latifolia	24	XX/XY	slightly smaller than autosomes (8% of female genome weight)	much longer than autosomes (9% of male genome weight)	M = 5.85 F = 5.73	3.5–24	Siroky et al. [2001] (Moore et al. [2003]; Nicolas et al. [2005]; Rautenberg et al. [2012])

[a] Putative X chromosome.

ship between the ages of sex chromosomes and the extent of Y/autosome or X/Y divergence. In terms of total genome size, *C. grandis* has the smallest genomes of all vascular plants with heteromorphic sex chromosomes (table 3).

Discussion

The Extent of Y/Autosome Divergence in C. grandis

Our results show that *C. grandis* has the greatest Y/autosome size difference documented in vascular plants (2.43 μm; table 1): The Y chromosome of *C. grandis* is 2.06 times larger than the largest chromosome (in contrast to previous reports of it being 2.5× or 3–4× longer than the largest autosome; Bhaduri and Bose [1947]; Guha et al. [2004]). Experimental work on *C. grandis*, using diploid individuals and artificial polyploids, has established the male-determining effect of the presence of the Y chromosome; individual tetraploid plants with a karyotype of XXXY still were normal males [Roy and Roy, 1971]. As previously reported, the chromosome number of *C. grandis* is 2n = 22 + XX or 22 + XY [Kumar and Deodikar, 1940 probably by mistake reported 2n = 26 for both sexes; Bhaduri and Bose, 1947; Chakravorti, 1948; Kumar and Vishveshwaraiah, 1952].

The degree of divergence of the male and female genome in *C. grandis* is evident also from the C-values: The difference between the male and female genomes is almost 0.1 pg of DNA, which is in the range of an entire small plant genome (*Genlisea margaretae*, 1C = 0.065 pg; Greilhuber et al. [2006]) and amounts to ca. 10% of the *C. grandis* genome (0.094 pg/2C). In *Silene latifolia*, the male genome weighs 5.85 pg/2C, the female 5.73 pg/2C, with the Y chromosome making up ca. 9% of the male genome and the X chromosome ca. 8% of the female genome [Siroky et al., 2001].

Autosome sizes in *C. grandis* vary from 2.28 to 1.44 μm in males and from 2.26 to 1.58 μm in females (table 1), both sexes having exclusively metacentric chromosomes (fig. 2), with the X chromosome probably the smallest chromosome of the complement, an assumption that needs testing. Both sexes also have the same number

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and distribution of rDNA sites on the autosomes (fig. 1C, F) while no rDNA site was detected on the Y chromosome. At least one of the chromosome pairs of *C. grandis* labeled with 45S rDNA bears a secondary constriction, but in contrast to previous reports [Bhaduri and Bose, 1947; Agarwal and Roy, 1984; Chattopadhyay and Sharma, 1991] no secondary constriction was seen on the Y chromosome. In species of *Silene*, *Rumex* and *Humulus* with heteromorphic sex chromosomes, the rDNA sites are also restricted to autosomes [Siroky et al., 2001; Karlov et al., 2003; Cuñado et al., 2007; Grabowska-Joachimiak et al., 2011], but *Spinacia oleracea* has a 45S rDNA site on the X chromosome [Lan et al., 2006]. It thus appears that rDNA does not greatly or regularly contribute to the morphological divergence of plant Y chromosomes.

Of the heteromorphic sex chromosomes that have been studied, most have undergone extensive rearrangements or end-to-end fusions. Thus, in *Podocarpus macrophyllus* (2n = 34 + X_1X_2Y; table 3), females have 38 telocentric chromosomes while males have 36 telocentric and 1 large submetacentric Y chromosome. In meiosis I, the *P. macrophyllus* Y chromosome pairs with 2 telocentric chromosomes to form a trivalent, suggesting it may have originated from a telocentric fusion of 2 telocentric chromosomes [Hizume et al., 1988]. In *Humulus japonicus*, a species with an XY_1Y_2 sex chromosome system (table 3), interstitial telomeric sites on 1 autosome pair also point to a fusion event having led to the reduction of the chromosome number from 18 to 14 + XY_1Y_2 [Grabowska-Joachimiak et al., 2011]. And in *S. latifolia* telomere-homologous sequences on the sex chromosomes provide evidence of a translocation of subtelomeric sites [Uchida et al., 2002]. In *C. grandis*, however, we did not find any telomeric sequences at interstitial sites (fig. 1B, E), suggesting that such fusions have not contributed, at least not recently, to the elongation of this species' Y chromosome.

Our GISH experiments revealed the preferential distribution of repetitive sequences in male and female individuals of *C. grandis*. In plants with small genomes, GISH signals tend to be unclear and restricted to pericentromeric heterochromatin blocks [Ali et al., 2004]. In *C. grandis* males, however, male and female genomic probes clearly differed in spite of the small genome size of the species (female individuals 0.849 pg/2C; male individuals 0.943 pg/2C). Male genomic DNA (fig. 1H) hybridized to centromeric and some subterminal regions of the chromosomes, while female genomic DNA (fig. 1I) hybridized mainly to centromeric regions. Both genomic probes hybridized to the Y chromosome, and C-banding results indicate that the Y chromosome is indeed mostly heterochromatic (fig. 4D, F). This fits with repetitive sequences forming large clusters in the centromeric and subterminal regions of the autosomes and having accumulated on the Y.

The types of repetitive DNA in the centromere of the *C. grandis* Y appear to be different from those in the centromeres of the autosomes and X chromosome (fig. 1I, inset). The situation might resemble that found in *S. latifolia*, where the centromeres of the autosomes and X chromosome are rich in *Silene* tandem repeat centromeric sequences and transposable elements, while the Y centromere contains *Silene* tandem repeat Y sequences [Cermak et al., 2008; Kejnovsky et al., 2009]. In *C. grandis* Y chromosomes, male-specific regions became progressively more visible with increasing concentrations of female blocking DNA (fig. 1J–L), and terminal regions that failed to label with either male or female DNA probably are pseudoautosomal regions, still engaged in recombination. In meiosis, there is an end-to-end connection between the X and the Y chromosome, but the X does not otherwise differ from the remaining chromosomes.

Ages of Plant Y Chromosomes and Their Size Change over Time

An increase in the size of some, but not all (table 3), vascular plant Y chromosomes has been attributed to the accumulation of repetitive DNA, especially transposable elements (Bergero et al. [2008], Cermak et al. [2008], Kejnovsky et al. [2009]: *Silene latifolia*; Mariotti et al. [2006, 2009], Cuñado et al. [2007]: *Rumex acetosa*). Such accumulation is thought to occur because of inefficient selection in non-recombining regions [Charlesworth and Charlesworth, 2000]. The best studied plant Y chromosome, that of *S. latifolia*, indeed does show signs of degeneration, including reduced levels of polymorphism, reduced gene expression levels, and transposable element insertion in Y genes [Filatov et al., 2000; Marais et al., 2008]. The degeneration, however, is less pronounced than that documented from animal sex chromosomes, perhaps because they are older or because of purifying selection during the haploid stage of the embryophyte life cycle [Armstrong and Filatov, 2008; Bergero and Charlesworth, 2011; Chibalina and Filatov, 2011]. An estimated 62% of the genes of *A. thaliana* are expressed in its haploid pollen tubes [Honys and Twell, 2003]. In liverworts, in which the haploid gametophyte is the predominant stage and in which there is no XX recombination, sex chromosome dimorphism may follow a different trajectory from that in vascular plants, where the diploid spo-

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rophyte is the predominant life phase (Yamato et al. [2007]; Bachtrog et al. [2011]; but see Jamilena et al. [2008] for the opposite view that the *Marchantia polymorpha* Y is in an advanced stage of degeneration, caused by the accumulation of a large amount of unique repetitive DNA sequences).

Comparing the speed of X/Y divergence is complicated by our poor understanding of the absolute ages of plant sex chromosomes. Thus, the sex chromosomes in the *S. latifolia* species group are approximately between 3.5 and 24 myr old. Synonymous site divergence values suggest ages of 8–24 myr [Moore et al., 2003] or 5–10 myr [Nicolas et al., 2005], while a phylogenetic study that used a relaxed molecular-clock approach instead inferred an age of the *Silene* clade with sex chromosomes of ca. 3.5 myr (Rautenberg et al. [2012] fig. 4: the node in question is the divergence of *S. latifolia* from *S. samia*). Molecular-clock work in liverworts is scarce, but judging from genetic branch lengths *Frullania* species are >2 myr old (Pleistocene; Bombosch et al. [2010]).

It has been hypothesized that the evolution of plant sex chromosomes may proceed from the initial recombination suppression and the expansion of the male-specific region through increasing heteromorphy between the X and Y chromosomes, followed by severe degeneration of the Y to its eventual loss (Ming et al. [2011] fig. 2). So far, there is no evidence for such a trajectory (table 3), and the limited data rather suggest that transposon accumulation and chromosome rearrangements occur idiosyncratically. It is clear, however, that plant sex chromosomes are all relatively young.

The sequencing and assembly of plant Y chromosomes is technically not yet feasible, and it is therefore unclear which transposon families they may accumulate. Nor is it clear in general how fast plant repetitive DNA is turned over [Renny-Byfield et al., 2011; Piednoel et al., 2012]. The only assembled Y chromosomes so far are those of *Homo sapiens* and chimpanzee [Skaletsky et al., 2003; Hughes et al., 2010]. However, once next-generation sequencing techniques yield longer read lengths, the relatively small genome of *C. grandis* compared to *S. latifolia* (c. 0.94 pg/2C vs. 5.85 pg; Costich et al. [1991]) and its phylogenetic proximity to the fully assembled crop species *C. sativus* [Huang et al., 2009] may make it a potentially useful additional system for the study of plant sex chromosomes.

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Chapter 6

General discussion

The main purpose of placing chromosome numbers in a phylogenetic context is to infer the direction of change that may have occurred during the course of evolution, from high to low numbers or the other way around. Until the turn of the millennium, cytogenetic studies did not explicitly consider phylogenetic relationships, and attempts to combine insights from microscopic studies with those from comparative (cladistic) frameworks were fraught with problems (cf. the Introduction of this thesis). Even with the availability of DNA phylogenies, the erroneous interpretation or use of sampled chromosome numbers for entire plant orders or families (e.g., Bedini et al., 2012) and the concept of a basic number ("x") inferred in *ad hoc* ways (Soltis et al., 2005), have persisted. In my doctoral research, I have contributed empirically as well as theoretically towards a new framework in which to think about the evolution of chromosome numbers.

Chromosome number and phylogenetics: How good is this combination?

The large monocot family Araceae was selected in this thesis to test the combination of cytogenetics and phylogenetics (Chapter 2, 3, and 4). The high frequency of $2n = 28$ in the well-counted clade Aroideae (Cusimano et al., 2012: Table S1; Sousa et al., 2014) probably unduly influenced early ideas about a (supposed) basic number x of 7 or 14 in the Araceae (Larsen, 1969; Marchant, 1973). These earlier hypotheses were developed before the relatively complete phylogenetic information for the Araceae that is available today, most importantly before the insight that the five genera of Lemnoideae (in the past treated as Lemnaceae) are nested inside the Araceae. The Lemnoideae have chromosome numbers of $n = 10$, 15, and 20, numbers that greatly affect the overall range of chromosome numbers found in early-diverging Araceae: The haploid numbers known so far are $n = 13$, 14, 15, 20, 24, and 30, and thus are relatively high. Using a phylogeny for the family that I enlarged to better cover certain chromosomally important groups and the approach developed by Mayrose and collaborators (2010), I found an evolutionary trend in the family from higher to lower chromosome numbers, rather than the other way around ($n = 16$ and 18: Chapter 2; $n = 16$: Chapter 4). The data also suggest a small role of polyploidization in the Araceae, different from many other groups of flowering plants.

Research on polyploidy in angiosperms began with Gates (1909) who discovered a tetraploid mutant of *Oenothera lamarckiana*, which exhibits larger cells and nuclei containing

28 instead 14 chromosomes. Just a few years earlier, P. Pernice (1889) had discovered the power of colchicine to inhibit microtubule formation, resulting in experimentally induced doubling of the entire complement of a cell's chromosome set, a method then perfected by Blakeslee (1939). With these tools in hand, interest in knowing and estimating how many plants are polyploid exploded. In 1994, Jane Masterson estimated, based on the relation between chromosome number and guard cell diameter that up to 80% of angiosperms may be polyploids. Using a different way of estimation, Wood et al. (2009) arrived at 31% in ferns and 15% in angiosperms. Such estimates, of course, greatly depend on the total number of angiosperm species that is assumed. This ranges from 304,419 accepted in *Plant List* (http://www.theplantlist.org/1.1/browse/A/#statistics vs. 1.1. of 13 Sep. 2013, accessed 12 Jan. 2014) to 352,000 (Patton et al., 2008). Earlier estimates were much lower and for many years the accepted number was 240,000 (Brumitt et al. 1992). A second, just as large, problem is that only a small fraction of angiosperms have had their chromosomes counted. Chromosome counts exist for 60,000 of the 300,000 to 352,000 species of flowering plants (Bennett, 1998; http://www.theplantlist.org/browse/A/), and many are listed in an electronic database for chromosome numbers, the 'Index of Plant Chromosome numbers' (http://mobot.mobot.org/W3T/Search/ipcn.html). Given the incomplete knowledge of angiosperm chromosome numbers, any percentages of polyploid angiosperms remain rough guesses.

Whatever the true fractions of polyploid species of ferns and flowering plants may turn out to be, only very few clades have had their history of polyploidization studied by the combination of cytogenetic and phylogenetic methods that is the *sine qua non* for inferring evolutionary direction (i.e. *Dahlia*: Gatt et al., 1999; *Nicotiana*: Chase et al., 2003; *Rhynchospora*: Vanzela et al. 2003; *Tragopogon*: *Soltis* et al., 2004; *Arabidopsis*: Lysak et al., 2006; *Tolmiea*, *Galax*, *Chamerion* , *Heuchera*, and *Vaccinium*: Soltis et al., 2007; *Trifolium*: Ansari et al., 2008; *Coffea*: Cenci et al., 2012). Much work is needed in this area.

As I have explained in the Introduction to this thesis and in Chapter 2, ancestral chromosome numbers today can no longer be inferred simply from published haploid or diploid numbers. A drastic example comes from the historically inferred "ancestral" haploid numbers in the Araceae. For example, if somebody would take my new Araceae counts from Chapter 3 and infer the basic number x in the traditional way, the result would be that Araceae have $x = 4$, this being the lowest reported count from the family. Other problems with the

concept of an ancestral "*x*" were discussed in the Introduction (pp. 5 and 6), among them the failure to consider information from outgroups.

The focus on the role of polyploidization in plant diversification has led to attempts to combine chromosome number, genome size, and phylogeny (e.g., Oyama et al., 2008; Sánchez-Jiménez et al., 2012; Soza et al, 2013; Pellicer et al., 2013; Pellicer et al., 2014). Several studies of this kind have disregarded that chromosome number and genome size are not evolutionary linked (Leitch and Bennett, 2004). The latter review showed for a sample of 546 monocots and 981 eudicots from many families that in many investigated cases, species with higher ploidy levels had smaller genome sizes than expected, in spite of their high chromosome numbers. The mechanism responsible for this empirical observation is genome downsizing, thought to be a common event, where hybridization is accompanied by extensive elimination of repetitive DNA and duplicated genes. This can completely mask the history of genomic and chromosome number change. For example, in maize, which underwent recent whole-genome duplication in addition to an ancient one, >50% of the duplicated genes have been deleted (Abrouk et al., 2010). Sometimes, the elimination of redundant DNA in a newly formed polyploid species is directional, leaving more of the DNA of one of the progenitors than of the other (Shaked et al., 2001; Chase et al., 2003). So far, only in ferns ($2n = 18$ to c.1440) is there a good linear relationship between chromosome number and genome size (Leitch and Leitch, 2012).

Phylogenetic modeling of chromosome number change, using the event-based approach of Mayrose et al. (2010), seems to be the best current manner of "reconstructing" ancestral chromosome numbers. At least this approach is reproducible. A caveat is that only with a dense sample of counted species, can we hope to arrive at solid inferences. In the Araceae, few chromosome counts are available for the outgroup families (see Chapter 4). Moreover, the outgroups are phylogenetically distant from the Araceae, which are the sister to a clade of all other Alismatales families, a divergence that is at least 120 million years old (Nauheimer et al., 2012). It is therefore not surprising that the long genetic branches and sparse counts near the base of the Araceae phylogeny result in great uncertainty for all inferred events near the root. However, the subsequent evolutionary downward trend in chromosome numbers is statistically well supported, going from $a = 16$ to 15 to 14 on the ultrametric tree and from $a = 16$ to 14 to 13 and back to 14 on the phylogram (Figs. 1 and S1 in Chapter 4).

Another problem is that there is no criterion for which depiction of the input phylogeny is preferable, a phylogram in which branch lengths are proportional to numbers of apomorphic substitutions or an ultrametric tree in which branch lengths are proportional to time, with time either relative (without a scale) or absolute (typically in million years). The two types of branch-length depiction can give similar or drastically different inference of chromosome numbers at internal nodes (Figs. 1 and S1 in Chapter 4). This problem is discussed in Cusimano and Renner (in review), who recommend carrying out inferences on both types of trees and then to use outside evidence to choose a preferred scenario. A good example showing this was the genus *Portulaca*, where ancestral chromosome numbers $a = 4$ or 5 were obtained on the phylogram but $a = 12$ on the ultrametric tree. Based on the observed chromosome number for this group, $a = 12$ is the more plausible result because it is similar to the chromosome numbers of the outgroups and because $n = 4$ is found in only one derived species while most others have numbers closer to $n = 12$. Cusimano and Renner (in review) also found that in some data sets, reconstructions are unaffected by the way branch lengths are modeled, and they suggest that simpler scenarios, explaining the data with fewer inferred steps, should probably be preferred. This might be one way to decide in cases where phylograms and ultrametric trees yield models of different complexity.

Molecular cytogenetic data support certain ancestral state reconstructions

A total of 29 species from 12 genera of Araceae were newly investigated in my cytogenetic work, and I used FISH with three DNA markers (5S and 45S rDNA, and *Arabidopsis*-like telomeres) on 24 of them and found new chromosome numbers in 21 (Chapters 3 and 4). The number of 5S rDNA sites (one) was conserved, and only in two species (*Cyrtosperma merkusii* with $2n = 39$ and *Englerarum hypnosum* with $2n = 24$) did I see atypical signals (see Chapter 4). However, the chromosomal distribution of 5S rDNA signals was highly variable among species (Chapters 3 and 4), and the number of 45S rDNA sites also varied. In the genus *Typhonium* (Chapter 3), most species (6) exhibited two 45S rDNA sites, with the exception of two that had polymorphic number of signals (five instead of four), one species with only one site, and other species with eight sites. On the other hand, 2 species of *Spathiphyllum* with $2n = 30$ had three or eight 45S rDNA sites (*S. pygmaeum* and *S. tenerum*), while an *Anthurium* species with the same chromosome number (*A. leuconerum*,

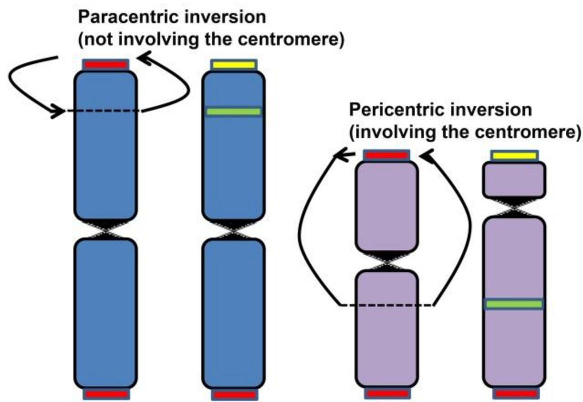
$2n$ = 30) had two sites, and another pair of close relatives, both with $2n$ = 60, had one or two 45S rDNA sites (*Monstera deliciosa* and *Scindapsus lucens*; Chapter 4). Telomeres were detected at the chromosome ends of all 24 species, and also in interstitial position in five species (*Typhonium laoticum* and *T.* spec. H.AR. 664: Chapter 3; *Anthurium leuconerum*, *A. wendlingeri*, and *Spathiphyllum tenerum*: Chapter 4).

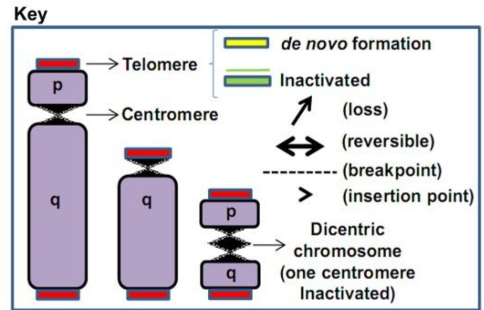
From these results it is clear that the power of the cytogenetic tools is that they point us (visually) to likely chromosome rearrangements that could have been responsible for the increase/decrease of chromosome numbers. My FISH results for the *Typhonium* genus (Chapter 3) supported three model-based ancestral chromosome number reconstructions: Two chromosome number reductions (by the observation of interstitial telomere repeats [ITR]) and one polyploidization event (higher number of 45S rDNA). Interstitial telomere repeats have been related mainly to three kinds of chromosome rearrangements (Fig. 1 reproduced here from Chapter 3, Fig. S5). For *T. laoticum* with $2n$ = 9, the existence of two interstitial telomere repeats (ITR) in the proximal region of the largest chromosome pair, seems to involve a different mechanism. I am explaining this by a reciprocal translocation between two acrocentric chromosomes, with one experiencing a break in its telomere sequence array and the other a break close to the centromeric region of its long arm. The products of this translocation would be a metacentric chromosome with a weakly detectible ITR, no longer functional, and a small chromosome comprising only part of the telomere sequence from one donor and the entire short arm and centromere of the other donor. Alternatively, only part of a telomere sequence might come from one donor and a centromere and the complete telomere sequence array from the other donor (Fig. 2 reproduced here from Chapter 3, Fig. 5).

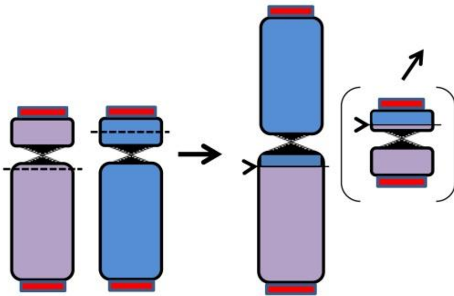
Similar events (Fig. 2) could have occurred in *Spathiphyllum tenerum* ($2n$ = 30) but hardly can explain the multiple signals found in *T.* spec. H.AR. 664 ($2n$ = 8), *Anthurium leuconerum* and *Anthurium wendlingeri* (both with $2n$ = 30). For these species, I am assuming a mechanism similar to what has been suggested for *Pinus* (Schmidt *et al.*, 2000). Telomere-like repeats are highly amplified in *Pinus elliottii* and not restricted to the ends of chromosomes; instead they form large intercalary and pericentric blocks, attributed to random short sequence arrays, perhaps extended by slippage replication, insertion of extra-chromosomal linear DNA fragments, or inversions (Biessmann and Mason, 1992). The high number of ITR discovered in *Anthurium leuconerum* and *A. wendlingeri* (Chapter 4) along

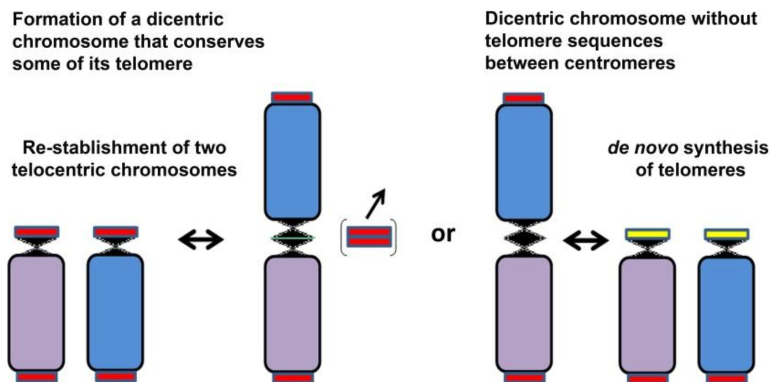
with the signal brightness must indicate huge repeat-amplifications, so far unlinked to obvious karyotype changes.

Fig. 1. (***next page***) Explanations proposed so far for the observation of interstitial telomere repeats. **a** paracentric or pericentric inversions: This kind of chromosome rearrangement does not imply a reduction in chromosome number. **b** chromosome fusion by symmetrical reciprocal translocation involving the centromere: This gives rise to a single chromosome and a small fragment composed mainly of the centromere of one chromosome and short rests of both previous chromosomes and their telomeres. Such short fragments will be eliminated from the cell unless they carry essential genes. **c** fusion-fission cycle or Robertsonian rearrangement: This involves a reciprocal translocation with breakpoints within the telomeric arrays of two telocentric chromosomes. This preserves both chromosomes' centromeres and telomere sequences although one of the centromeres and the interstitial telomeric sequences must be inactive. **a** modified from Schubert (2007) and **b** and **c** from Schubert and Lysak (2011).


a) Interstitial telomeric sites as a result of chromosomal inversions
Paracentric inversion
(not involving the centromere)
Pericentric inversion
(involving the centromere)


Key
Telomere
Centromere
p
q
de novo formation
Inactivated
(loss)
(reversible)
(breakpoint)
(insertion point)
Dicentric
chromosome
(one centromere
Inactivated)


b) Chromosome fusion by symmetrical reciprocal translocation
(involving the centromere)


c) Fusion-fission cycle or Robertsonian rearrangements
Asymmetric reciprocal translocation
(not involving the centromere)
Formation of a dicentric
chromosome that conserves
some of its telomere
Dicentric chromosome without
telomere sequences
between centromeres
Re-stablishment of two
telocentric chromosomes
or
de novo synthesis
of telomeres

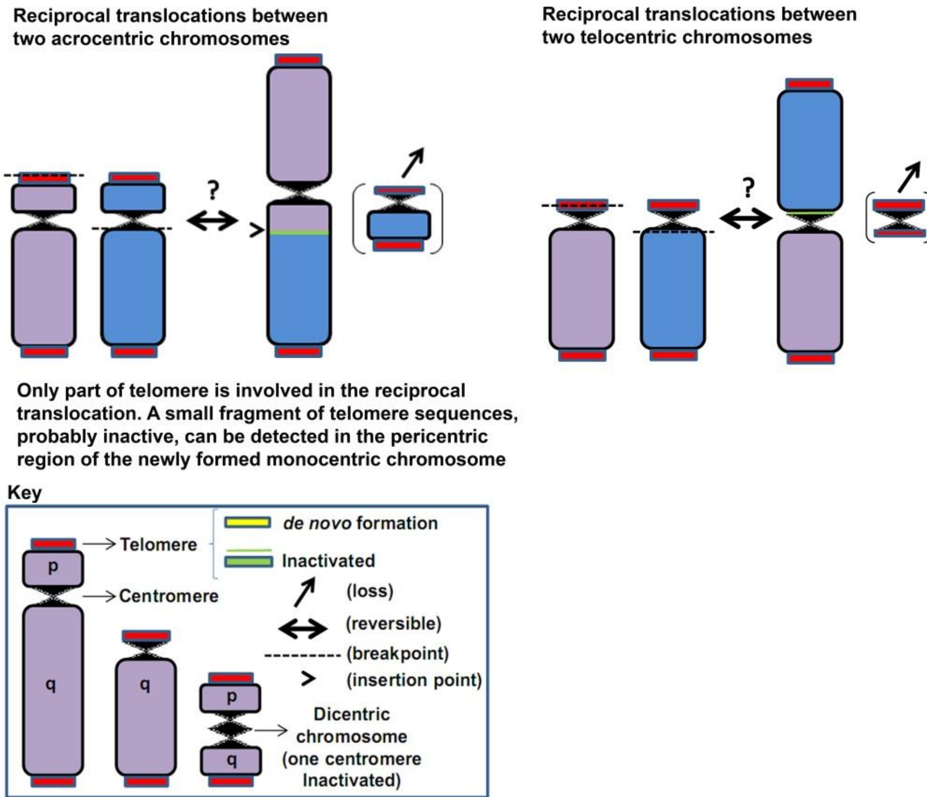


Fig. 2. Proposed explanation for the observed ITR in the proximal region of the largest chromosome pair of *T. laoticum*. It assumes a reciprocal translocation between two acrocentric chromosomes with one chromosome having breaks in its telomere sequence array and the other close to the centromeric region of its long arm. The product of this translocation would be a metacentric chromosome (monocentric) with a weakly detectible ITR, no longer functional, plus a small chromosome comprising only part of the telomere sequence from one donor and the entire short arm and centromere of the other donor. Alternatively, only part of a telomere sequence might come from one donor and a centromere and complete telomere sequence array from the other donor.

Interstitial telomere repeats are not always related to chromosome fusions

Interstitial telomere repeats (ITRs) are rare in plants, but are known from *Vicia faba* (Schubert et al., 1995; Fuchs et al., 1995: Fig. 1), *Othocallis siberica* (Weiss-Schneeweiss et al., 2004), *Eleocharis subarticulata* (Da Silva et al., 2005), *Sideritis montana* (Raskina et al., 2008), and two species of *Typhonium* (Sousa et al., 2014). In *Vicia faba*, presence of ITRs was related to the existence of fusion-fission cycles, and in *Typhonium* to Robertsonian-

fusion-like rearrangements. The Robertsonian (Rb) fusion, a chromosome rearrangement involving centric fusion of two acro-(telo)centric chromosomes to form a single metacentric, is thought to be frequent in mammals (Slijepcevic, 1998).

The best characterization of ITRs comes from studies on the human genome. Using FISH, Azzalin et al. (2001) detected multiple such interstitial telomere arrays (over 50) on human chromosomes, a finding difficult to explain by tandem fusion. In their investigation, three different classes of ITR were identified and characterized as (i) short ITR, composed of few, essentially exact vertebrate telomere repeat $(T_2AG_3)_n$, (ii) subtelomeric ITR, composed of larger arrays (several hundred base pairs) including many degenerate units within subtelomeric domain units, and (iii) fusion ITR, in which two extended stretches of telomeric repeats are oriented head-to-head. Without actual sequence information, low copy telomere repeats nested in long terminal repeat elements in subterminal regions can be misinterpret as a fusion site, as demonstrated by Azzalin et al. (2001) using the 1477 bp fragment from clone p20. This study makes clear that many mechanisms are involved in the formation of the three classes of ITR in humans alone, and only 2 of the 50 ITRs likely originated by the fusion of two ancestral chromosomes (Azzalin et al., 2001). In the grass species *Aegilops tauschii*, genomic analyses identified 27 major translocation breakpoints, of which nine were fusions involving end-to-end / telomere-telomere sequence (Luo et al., 2009). The latter, however, was never cytologically tested using FISH.

Another example of the disconnection between interstitial telomere sites and past chromosome fusion is *Othocallis siberica* (Weiss-Schneeweiss et al., 2004). When the typical telomere repeat of plants $(T_3AG_3)_n$ was hybridized to the chromosomes of this species, only two signals were detected: one terminal and one ITR in distinct chromosomes (2 and 4). After hybridization with the telomere vertebrate repeat $(T_2AG_3)_n$, it turned out that all terminals of the chromosomes matched this sequence, while the two interstitial/telomere repeats matched the *Arabidopsis*-like telomere probe and $(T_2AG_3)_n$, forming large mixed blocks. Regardless of the mechanism(s) of its origin (double strand break repair by telomerase in the germ line of the cell, chromosome integration of extra-chromosomal segments via transposons carrying telomere sequences, or introgression via hybridization), the coexistence of these telomere repeats (vertebrate and *Arabidopsis*-like) in *O. siberica* and the observation of ITR composed of *Arabidopsis*-like telomere repeat in one chromosome together suggest that the latter may be

the remnants of ancestral genome rearrangements that occurred before the switch to vertebrate-type telomeric repeats (Weiss-Schneeweiss et al., 2004).

Although the visualization of interstitial telomere repeats detected by FISH can be interpreted as fusions sites, more studies on micro-dissection, cloning, sequencing and characterization are essential to fully understand their origin. Such studies so far have only been carried out for the human genome (Azzalin et al., 2001). Future work will require applying similar approaches as used to study the human karyotype in plants to classify and describe in detail the ITR sequences and to then undertake comparative analyses.

Insights into the sex chromosomes of *Coccinia grandis* (Curcubitaceae)

The last Chapter (5) of this thesis contains the first molecular-cytogenetic study in *Coccinia grandis*, a dioecious species with heteromorphic sex chromosomes. Although important studies in the 1950s and 1970s had established the male-determining effect of the presence of the Y chromosome (Kumar and Viseveshwaraiah, 1952; Roy and Roy, 1971), prior to my work the size of the *C. grandis* genome and details of its karyotype were unknown. Cytological photographs of *C. grandis* ♂ in Roy and Roy (1971) revealed a large Y chromosome with primary (centromere) and secondary (NOR) constrictions. With the goal of investigating if rDNA sequences could be involved in the lengthening of the Y chromosome and wanting to document the distribution of the repetitive DNA in the *C. grandis* genome, I analyzed male and female individuals with FISH (5S and 45S rDNA, and *Arabidopsis*-like telomeres), GISH, and C-banding techniques.

In the initial karyotype analysis, no secondary constriction was found on the Y chromosome, an observation supported by my FISH results. However, secondary constrictions were seen in one autosomal chromosome pair in mitotic metaphase and represent active nucleolar organizing regions (NORs), both are 45S rDNA-positive when detected by FISH. The absence of an rDNA site on the Y chromosome (judging by FISH) fits with its lack of a secondary constriction. In species of *Silene*, *Rumex* and *Humulus* with heteromorphic sex chromosomes, the rDNA sites also are confined to autosomes (Siroky et al., 2001; Karlov et al., 2003; Cuñado et al., 2007; Grabowska-Joachimiak et al., 2011), but *Spinacia oleracea*, which has homomorphic sex chromosomes, has a 45S rDNA site on the X chromosome (Lan et al., 2006). Based on these data, it appears that rDNA does not greatly

contribute to the morphological divergence of plant Y chromosomes. The distribution of telomere signals was restricted to the chromosome ends in all tested male and female individuals of *C. grandis*, suggesting that chromosome fusions also have not contributed, at least not recently, to the elongation of the Y chromosome (Sousa et al., 2013; Chapter 5).

The genomic *in situ* hybridization (GISH) showed two preferential distributions of repetitive DNA related to the sex. Male genomic DNA hybridized to centromeric and in some subterminal regions of the chromosomes, while female genomic DNA hybridized mainly to centromeric regions (Chapter 5). Both genomic probes hybridized to the Y chromosome, and C-banding results indicate that the Y chromosome is indeed mostly heterochromatic. This fits with repetitive sequences forming large clusters in the centromeric and subterminal regions of the autosomes and having accumulated on the Y. Interestingly, the type of repetitive DNA in the centromere of the *C. grandis* Y chromosome appears to be different from those in the centromeres of the autosomes/X chromosome. The Y centromeric region is not well labeled with male or female genomic probes and clearly differs from the dot signals seen in the centromeres of the remaining chromosomes of this species. In *Silene latifolia*, the centromeres of the autosomes/X chromosomes are rich in *Silene* TAndem Repeat Centromeric (STAR-C) sequences and transposable elements, while the Y centromere contains *Silene* TAndem Repeat Y (STAR-Y) chromosome sequences and transposable elements (Cermak et al., 2008; Kejnovsky et al., 2009).

Analysis of the C-value of *C. grandis* revealed a male/female genome difference of almost 0.1 pg of DNA, which is in the range of an entire plant genome (*Genlisea margaretae*, 1C = 0.065 pg; Greilhuber et al., 2006). This difference in fact amounts to some 10% of the *C. grandis* genome (0.094 pg/2C). In *Silene latifolia*, the male genome weighs 5.85 pg/2C, the female 5.73 pg/2C, with the Y chromosome making up c. 9% of the male genome and the X chromosome c. 8% of the female genome (Siroky et al., 2001). Today, the sequencing and assembly of plant genomes without a closely related reference genome is technically feasible (Chamala et al., 2014), but *C. grandis* is closely related to the fully assembled species *Cucumis sativus*, *Cucumis melo, Cucumis hystrix* and *Citrullus lanatus* (Huang et al., 2009; Garcia-Mas et al., 2012; Guo et al., 2013; Yang et al., 2014) Therefore, a genomic approach could be used in *C. grandis* to identify sex chromosomal markers. My results have revealed that the Y chromosome in *C. grandis* is heterochromatic, similar to the Y chromosomes of *Rumex acetosa*, and thus different from the euchromatic Y chromosome of *Silene latifolia*; it

is more than two times larger than the largest chromosome in the genome; and its small genome (above) makes *C. grandis* and its con-generic species without heteromorphic sex chromosomes ideal system for sequencing and studying the molecular steps of sex chromosome differentiation in land plants.

General conclusions

The results of my doctoral research (Chapter 2, 3 and 4) contribute to our understanding of the evolution of plant chromosomes. Specifically, I combined molecular-cytogenetic approaches with phylogenetic analytical approaches. I also produced new empirical data relevant for the phylogenetics of Araceae, their chromosome numbers and karyotypes, and the karyotype of *Coccinia grandis*, the angiosperm with the largest known XY size difference (Chapter 5). The next step towards a deeper understanding of chromosomal evolution in these clades now requires the addition of full genome sequencing and bioinformatics to the molecular-cytogenetic and phylogenetic approaches used here.

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