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DISSERTATION

Molecular phylogeny and chromosome evolution of the genus *Melampodium* L. (Millerieae, Asteraceae)

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Mag. Cordula Blöch

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TABLE OF CONTENTS

DESCRIPTION OF THE CONTRIBUTION TO THE INDIVIDUAL MANUSCRIPTS 7

INTRODUCTION 9

MOLECULAR PHYLOGENETIC ANALYSES OF NUCLEAR AND PLASTID DNA SEQUENCES SUPPORT DYSPLOID AND POLYPLOID CHROMOSOME NUMBER CHANGES AND RETICULATE EVOLUTION IN THE DIVERSIFICATION OF *MELAMPODIUM* (MILLERIEAE, ASTERACEAE) 19

RECONSTRUCTING BASIC CHROMOSOME NUMBER EVOLUTION IN THE GENUS *MELAMPODIUM* (ASTERACEAE).....57

REPEATED CYCLES OF HYBRIDIZATION AND POLYPLOIDIZATION IN *MELAMPODIUM*: ORIGIN AND GENOME EVOLUTION OF ALLOPOLYPLOIDS OF SECT. *MELAMPODIUM* (ASTERACEAE).....83

CONCLUSIONS 135

ABSTRACT 139

ZUSAMMENFASSUNG 141

APPENDIX: NEW CLASSIFICATION OF THE GENUS *MELAMPODIUM*.....143

CURRICULUM VITAE 146

DESCRIPTION OF THE CONTRIBUTION TO THE INDIVIDUAL MANUSCRIPTS

Manuscript 1: Molecular phylogenetic analyses of nuclear and plastid DNA sequences support dysploid and polyploid chromosome number changes and reticulate evolution in the diversification of *Melampodium* (Millerieae, Asteraceae)
C. Blöch, H. Weiss-Schneeweiss, G.M. Schneeweiss, M.H.J. Barfuss, C.A. Rebernig, J.L. Villaseñor, T.F. Stuessy

- Collecting of the plant material (silica-gel leaf material, seeds, fixation of flower buds) in Mexico and the USA (field trips 2006-2007; with Prof. Dr. T.F. Stuessy, Dr. J.L. Villaseñor, and Dr. C.A. Rebernig).
- Co-planning of the experiments (with Prof. Dr. T.F. Stuessy and Dr. H. Weiss-Schneeweiss).
- Most of the laboratory works (partly with the technical assistant): DNA extraction, DNA cloning and sequencing.
- Data analyses (in collaboration with and under supervision of Dr. G. Schneeweiss).
- GenBank submission of the sequence data
- Relevant literature survey; figure preparation; drafting and co-writing of the manuscript.

Manuscript 2: Reconstructing basic chromosome number evolution in the genus *Melampodium* (Asteraceae)
C. Blöch, H. Weiss-Schneeweiss, G.M. Schneeweiss, T.F. Stuessy

- Collecting of the plant material in Mexico and the USA (field trips 2006-2007; with Prof. Dr. T.F. Stuessy and Dr. J.L. Villaseñor).
- Co-planning of the analyses (with Prof. Dr. T. Stuessy and Dr. G.M. Schneeweiss).
- Relevant literature survey; figure preparation; drafting and co-writing of the manuscript.

Manuscript 3: Repeated cycles of hybridization and polyploidization in *Melampodium*: origin and genome evolution of allopolyploids of sect. *Melampodium* (Asteraceae).
C. Blöch, H. Weiss-Schneeweiss, G.M. Schneeweiss, B. Rupp, J.L. Villaseñor, T.F. Stuessy

- Collecting of the plant material (silica-gel leaf material, seeds, fixation of flower buds) in Mexico and the USA (field trips 2006-2007; with Prof. Dr. T.F. Stuessy and Dr. J.L. Villaseñor).
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INTRODUCTION

This doctoral thesis was designed to investigate the phylogeny of the genus *Melampodium* and to provide a robust framework for analyses of chromosomal numerical (dysploidy and polyploidy) and structural changes (origin and evolution of selected allopolyploids) in the genus which encompasses 40 species exhibiting a wide variation of basic and haploid chromosome numbers.

Molecular phylogeny – Developed in recent decades, molecular phylogenetic techniques help to refine and test previous classifications largely based on morphology and allow better insight into plant relationships on different classification levels (e.g., Chase & al., 1993; Bayer & al., 1996) as well as on populational levels (e.g., Rebernig & al., 2010). Biomathematics has added to the improvement by offering methods, which make it possible to analyse these characters with phenetic (e.g., neighbour-joining), cladistic (e.g., maximum parsimony), or likelihood based methods (e.g., maximum likelihood and closely related Bayesian analysis; Felsenstein, 2004).

Phylogenetic studies of wild plant groups are most often based on selected plastid DNA sequences and/or nuclear ribosomal internal transcribed spacer (ITS) region sequence data. Chloroplast DNA has the advantage of being generally structurally stable, haploid, non-recombinant, and uni-parentally inherited (for review, see Palmer, 2005). Nuclear markers are biparentally inherited, which renders them very informative for revealing hybridization events. Nuclear ITS data are usually easily collected, but their interpretation suffers from interference of processes such as concerted evolution (homogenization of individual rDNA repeats) or gene conversion (Àlvarez & Wendel, 2003). Nuclear low-copy genes are better suited to investigate evolution of the groups involving hybridization/polyploidization than ITS (Raymond & al., 2002; Sang, 2002; Àlvarez & Wendel, 2003; Hughes & al., 2006; Kim & al., 2008), although they are more expensive and labour-intensive. A reliable phylogeny of a genus

can further be used to test various character evolutions within this group (e.g., Vanderpoorten & Goffinet, 2006; Mayrose & al., 2010). Plastid and nuclear phylogenies can also be used to test the occurrence of hybridization both on homoploid and polyploid level (Ferguson & Sang, 2001; Kim & al., 2008).

Chromosome number evolution & hybridization – The importance of chromosomal change in the evolution of vascular plants is undeniable (Stebbins, 1971; Grant, 1981; Levin, 2002; Guerra, 2008), although the direct role of chromosomal change in speciation remains controversial (Rieseberg, 2001; Ayala & Coluzzi, 2005). Chromosomal change, particularly involving change in number, may act as a barrier to gene flow and blur or complicate the relationships between taxa analysed (Guerra, 2008; Navarro and Barton, 2003).

Dysploid chromosome number change – Dysploidy, the "stepwise increase or decrease in the haploid chromosome number observed among related species, often forming dysploid series" (Ehrendorfer, 1964), is rather common in relatively closely related plant groups. Although the role and consequences of dysploid chromosome number change in plant evolution are still not thoroughly understood, it is widely acknowledged that such changes may confer effective reproductive isolation (Grant, 1981). The analyses aiming at inferring the direction and mechanisms of dysploidy in various plant groups require a good hypothesis on the relationships within the groups (either based on morphology, or even better, on molecular phylogenetic data) and detailed information on chromosome numbers of all (or most) taxa in the group (Guerra, 2008). Basic chromosome numbers may either be correlated to phylogeny of the group and thus be a delimiting character for the classification of these taxa (e.g., *Passiflora*/Passifloraceae, Hansen & al., 2006; *Pennisetum*/Poaceae, Martel & al., 2004; *Rhaponticum*/Asteraceae and related genera, Hidalgo & al., 2007) or may be uninformative for phylogenetic relationships (*Crepis*/Asteraceae, Enke &

Gemeinholzer, 2008; *Trifolium*/Fabaceae: Ellison & al., 2006; *Carex*/Cyperaceae, Hipp & al., 2009). Phylogenetically indicative chromosome numbers may form ascending, descending or a mixed (combination of ascending and descending) dysploid series. The most apparent dysploid chromosome number change is caused by Robertsonian exchanges (fission or fusion; Jones, 1998). However, these processes may be blurred by other structural karyotype rearrangements that have accompanied or followed the evolution of the group (Jones, 1998; Lysak & al., 2006).

Hybridization & polyploidy – Hybridization and polyploidization (Whole Genome Duplication; WGD) are ubiquitous in plants and their frequency suggests that they may confer selective advantage (Stebbins, 1971; Grant, 1981; Rieseberg, 2001; Levin, 2002; Comai, 2005). Both processes can either act alone resulting in autopolyploids or homoploid hybrids (e.g., Rieseberg, 1991; Ferguson & Sang, 2001; Soltis & al., 2007; Parisod & al., 2010), respectively, or in concert producing allopolyploids, i.e., hybrids with fully duplicated genomes (e.g., Pires & al., 2004; Adams & Wendel, 2005; Kim & al., 2008; Tate & al., 2009). While traditional estimates of the frequency of polyploidy among angiosperms vary between 30% and 80% (Masterson, 1994), recent studies indicate that most of angiosperms have undergone polyploidization at least once in their evolutionary history (Soltis & al., 2009). Polyploidy has been estimated to be involved in 2-15% of plant speciation events (Otto & Whitton, 2000; Wood & al., 2009). Regardless of the estimate, polyploidy and hybridization are recognized as a major force in the evolution of angiosperms, allowing and promoting, e.g., subfunctionalization and neofunctionalization of genes, gene loss, epigenetic changes affecting gene expression, transposable element activation, and larger genome rearrangements (Adams & Wendel, 2005; Le Comber & al., 2010; Parisod & al., 2010). Established polyploids undergo genome diploidization often manifested in, e.g., chromosomal rearrangements or genome downsizing (Clarkson & al., 2005; Tate & al., 2009; Le Comber & al., 2010).

Genus *Melampodium* – *Melampodium* (Millerieae, Asteracaeae) is a medium-sized genus with 40 annual and perennial species centered in tropical and subtropical Mexico and Central America with six species in the adjacent southwestern United States and three species in Colombia and Brazil (Stuessy, 1972). *Melampodium* is closely related to two small genera *Acanthospermum* and *Lecocarpus*, which were postulated to have been derived from within *Melampodium* (Stuessy, 1972). All species of *Melampodium* are tap-rooted except for two species in sect. *Rhizomaria*, and all are yellow-rayed, except for three species of the white-rayed complex (ser. *Leucantha*, sect. *Melampodium*). Stuessy (1972) in the latest taxonomic treatment of the genus recognized six sections and subdivided the largest section into five series. These relationships were tested and largely supported by phenetic and cladistic analyses of morphological characters (Stuessy, 1979; Stuessy & Crisci, 1984).

Chromosome numbers have long been recognized as important characters in the evolution of *Melampodium* (Turner & King, 1961; Stuessy, 1971, 1972, 1979; Weiss-Schneeweiss & al., 2009). The genus displays a wide variation of haploid chromosome numbers (n = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33), which are derived from five basic chromosome numbers (x = 9, 10, 11, 12 and 14; Stuessy, 1971; Weiss-Schneeweiss & al., 2009). Previous studies suggested x = 10 (sect. *Melampodium*) as the ancestral chromosome base number for the genus due to its presence in more than 50% of the species and due to the presence of putatively ancestral type of sterile disc ovaries in this group (Stuessy, 1971). Chromosome numbers have been used as important delimiting characters in the most recent classification of the genus with four of the six sections recognized by Stuessy (1972) having a unique chromosome number and two sharing a common chromosome number (Stuessy, 1972; Weiss-Schneeweiss & al., 2009).

Polyploidy (both on tetraploid and hexaploid level) is known in 16 species of *Melampodium* (Stuessy, 1971; Stuessy & al., 2004; Weiss-Schneeweiss & al., 2009). Both autopolyploidy (*Melampodium aureum*, and tetraploid cytotypes of *M. cinereum* and *M. leucanthum*, Stuessy, 1971, Stuessy & al., 2004) and allopolyploidy (*M. sericeum*, Stuessy, 1971; *M. paniculatum*, Stuessy & Brunken, 1979) have been suggested as a mode of polyploid origin.

Aims – This PhD thesis is divided into three chapters, each presented as a paper (either published or in preparation). Two plastid markers (the *mat*K gene and the *psb*A-*trn*H spacer), and three nuclear markers (ITS, 5S rDNA spacer, *Pgi*C[1] low copy nuclear gene) have been employed in different combinations to analyse the phylogenetic relationships in the genus, to infer the mode of basic chromosome number change, and to test the origin of polyploid taxa. Furthermore classic and molecular karyotype analysis, genome size, and ITS restriction patterns were investigated in polyploids and related diploids.

1) Molecular phylogenetic analysis of nuclear and plastid DNA sequences support the important roles of dysploid and polyploid chromosome number changes as well as of reticulate evolution in the diversification of *Melampodium* (Millerieae, Asteraceae).

   Molecular phylogenetic analyses of the plastid gene *mat*K and of the nuclear ribosomal ITS region of all the species of the genus have been employed to analyse the phylogenetic relationships within the genus and to test the previous classification of Stuessy (1972). The study has aimed to answer following questions: (1) What are the phylogenetic relationships among *Melampodium*, *Acanthospermum* and *Lecocarpus*, and is *Melampodium* monophyletic? (2) How well does the current taxonomic classification (Stuessy, 1972) reflect phylogenetic relationships among

[1] encodes the cytosolic isozyme of phosphoglucose isomerase.

the species? (3) Are the chromosome base numbers predictive of evolutionary lineages? (4) Which mode of polyploidization (auto- vs. allopolyploidy) is the most common in the genus, and which parental species have been involved in these events?

2) Reconstructing basic chromosome number evolution in the genus *Melampodium* (Asteraceae).

The second chapter presents the analyses of the directionality of basic chromosome number evolution in *Melampodium* and aims to reconstruct the ancestral chromosome number of the genus, which has earlier been postulated to be $x = 10$ (Turner & King, 1961; Stuessy, 1971). Plastid and nuclear phylogenies have been used as framework for ancestral character state reconstruction. Specifically, the following questions were addressed: (1) Is the basic chromosome number distribution in *Melampodium* indicative of descending, ascending, or mixed type of dysploidy? (2) Have the different basic chromosome numbers of the genus evolved once or recurrently? (3) What is the reconstructed ancestral basal chromosome number of the genus?

3) Repeated cycles of hybridization and polyploidization in *Melampodium*: origin and genome evolution of allopolyploids of sect. *Melampodium* (Asteraceae).

Six polyploid species of sect. *Melampodium* hypothesized to be of allopolyploid origin based on karyotypic analyses and/or incongruencies between plastid and nuclear phylogenies have been studied to unambiguously infer their mode of origin and identify the putative parental taxa. Furthermore, genome rearrangements accompanying evolution of the polyploids have been studied employing phylogenetic analyses of several plastid and nuclear markers, ITS restriction pattern analyses, rDNA loci localization in chromosomes with FISH, and genome size measurements. Specifically, the following questions have been investigated: (1)

What is the mode of the origin of the six polyploid species, and which putative parental taxa were involved? (2) Which type of changes have accompanied hybridization and polyploidization on genomic, chromosomal, and sequence levels? (3) Are there parallels in the genome evolution in two closely related allopolyploid taxa of the same parental origin, *M. sericeum* and *M. pringlei*? (4) What is the role of reticulate evolution for speciation in sect. *Melampodium*?

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MOLECULAR PHYLOGENETIC ANALYSES OF NUCLEAR AND PLASTID DNA SEQUENCES SUPPORT THE IMPORTANT ROLES OF DYSPLOID AND POLYPLOID CHROMOSOME NUMBER CHANGES AND RETICULATE EVOLUTION IN THE DIVERSIFICATION OF *MELAMPODIUM* (MILLERIEAE, ASTERACEAE)

Cordula Blöch[1], Hanna Weiss-Schneeweiss[1], Gerald M. Schneeweiss[2], Michael H.J. Barfuss[1], Carolin A. Rebernig[1], José L. Villaseñor[3] & Tod F. Stuessy[1]

[1]Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

[2]Department of Biogeography and the Botanical Garden, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

[3]Departamento de Botánica, Instituto de Biologia, Universidad Nacional Autónoma de México, México, D.F., Mexico

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Abstract

Chromosome evolution (including polyploidy, dysploidy, and structural changes) as well as hybridization and introgression are recognized as important aspects in plant speciation. A suitable group for investigating the evolutionary role of chromosome number changes and reticulation is the medium-sized genus *Melampodium* (Millerieae, Asteraceae), which contains several chromosome base numbers (x = 9, 10, 11, 12, 14) and a number of polyploid species, including putative allopolyploids. A molecular phylogenetic analysis employing both nuclear (ITS) and plastid (*mat*K) DNA sequences, and including all species of the genus, suggests that chromosome base numbers are predictive of evolutionary lineages within *Melampodium*. Dysploidy, therefore, has clearly been important during evolution of the group. Reticulate evolution is evident with allopolyploids, which prevail over autopolyploids and several of which are confirmed here for the first time, and also (but less often) on the diploid level. Within sect. *Melampodium*, the complex pattern of bifurcating phylogenetic structure among diploid taxa overlain by reticulate relationships from allopolyploids has non-trivial implications for intrasectional classification.

Keywords:

Asteraceae, dysploidy, ITS, *mat*K, *Melampodium*, phylogeny, polyploidy, reticulate evolution.

1. Introduction

Chromosome evolution, involving both numerical (polyploidy and dysploidy) and structural changes (e.g., inversions, translocations), as well as hybridization and introgression, are recognized as important aspects of plant speciation (Rieseberg, 2001; Schubert, 2007; Leitch and Leitch, 2008). A requisite for assessing the role of chromosomal change in a given group is to have a sound hypothesis of the group's phylogeny (Rieseberg, 2001). It is important to know whether chromosome base numbers are correlated with phylogenetic lineages, as is sometimes the case (e.g., Schneeweiss et al., 2004a, b; Hansen et al., 2006; Hidalgo et al., 2007), or whether they are independent (e.g., Baldwin and Wessa, 2000; Mast et al., 2001; Yuan et al., 2004; Ellison et al., 2006). This allows their causative role in diversification to be interpreted properly. Molecular data can provide precise estimates of phylogenetic relationships as well as evidence concerning taxa involved in hybridization at both the diploid and the polyploid level. Examples of such studies include *Achillea* (Guo et al., 2004, 2006), *Glycine* (Doyle et al., 2003), *Helianthus* (Rieseberg, 1991; Rieseberg et al., 2007), *Nicotiana* (Lim et al., 2004), and *Paeonia* (Ferguson and Sang, 2001).

A suitable group for investigating the evolutionary role of chromosome number changes and reticulation is the genus *Melampodium* (Asteracaeae). It is medium-sized and comprises 40 annual and perennial species (Stuessy, 1972; Turner, 1988, 1993, 2007) centered in tropical and subtropical Mexico and Central America with five species distributed in the adjacent southwestern United States and three species scattered in Colombia and Brazil. With the exception of the only recently described *M. moctezumum* (Turner, 2007), all species have now been counted chromosomally and the following haploid chromosome numbers have been reported (Stuessy, 1968, 1970b,

1971, 1972; Keil and Stuessy, 1975, 1977; H. Weiss-Schneeweiss et al., unpubl.[1]): *n* = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33. *Melampodium* is closely related to *Acanthospermum* (six species in the Americas and on the Galapagos Islands; Stuessy, 1970a) and *Lecocarpus* (three to four species endemic to the Galapagos Islands; Elliasson, 1971; Adsersen, 1980; Sønderberg Brok and Adsersen, 2007), with which it shares functionally staminate disk florets and pistillate ray florets as well as inner phyllaries (involucral bracts) each tightly enclosing and fused with a single ray achene (Stuessy, 1970a). The generic distinctness of these groups, which together constitute a generic complex classified as a separate subtribe Melampodiinae (Hoffmann, 1890; Panero, 2007), only recently moved from tribe Heliantheae s.s. to tribe Millerieae (Panero, 2007; Baldwin, in press), has never been seriously doubted. It has been suggested, however, that *Acanthospermum* and *Lecocarpus* might have been derived from within *Melampodium* (Stuessy, 1971).

A previous intuitive phylogenetic hypothesis (Stuessy, 1972), which was tested by cladistic (Stuessy, 1979) and phenetic (Stuessy and Crisci, 1984) analyses of morphological characters, suggested that basic chromosome numbers correspond well with delimitation of sections. Four sections have unique chromosome base numbers (sections *Zarabellia*, *Melampodium*, *Serratura*, and *Bibractiaria* with *x* = 9, 10, 12, and 14, respectively), whereas two (sections *Alcina* and *Rhizomaria*) share *x* = 11 (Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.). Dysploidy is not restricted to the diploid level but also occurs at the polyploid level as evidenced by *n* = 23 derived from *n* = 12 in *M. dicoelocarpum* (Stuessy, 1971).

Stuessy (1971) proposed *x* = 10 as the ancestral chromosome base number in the genus because it is found in the morphologically highly variable and most species-rich

[1] Weiss-Schneeweiss, H., Villaseñor, J.L., Stuessy, T.F., 2009**.** Chromosome numbers, karyotypes, and evolution in *Melampodium* (Asteraceae). Int. J. Pl. Sci. 170, 1168–1182.

sect. *Melampodium* (hence divided into the five series *Cupulata*, *Leucantha*, *Longipila*, *Melampodium*, and *Sericea*; Stuessy, 1972), and correlates with occurrence of the presumably primitive type of conspicuous and clearly differentiated sterile ovary of the functionally male disk florets (Stuessy, 1972), otherwise found in *Acanthospermum* and *Lecocarpus*. The other chromosomal lines, which share the presumably derived character of disk florets with diminutive and undifferentiated sterile ovaries, were suggested to be derived from $x = 10$ by either loss ($x = 9$) or gain ($x = 11$ and $x = 12$) of chromosomes (Stuessy, 1971). In conflict with the above hypothesis, however, is the presence of $x = 11$ in the related genera *Acanthospermum* and *Lecocarpus* (Stuessy, 1971; Keil et al., 1988; H. Weiss-Schneeweiss, unpubl.).

Polyploidy (both on tetraploid and hexaploid levels) has played an important role in diversification of *Melampodium* with polyploidy being known in 16 species (40% of the genus). Of those, seven are uniformly tetraploid and five uniformly hexaploid, whereas intraspecific cytotype mixtures of diploid and tetraploid cytotypes and of tetraploid and hexaploid cytotypes are known from three and one species, respectively (Stuessy, 1971; Stuessy et al., 2004; H. Weiss-Schneeweiss et al., unpubl.). Among polyploids, both autopolyploid (*M. aureum*, and tetraploid cytotypes of *M. cinereum* and *M. leucanthum*, Stuessy, 1971, Stuessy et al., 2004) and allopolyploid (*M. sericeum*, Stuessy, 1971; *M. paniculatum*, Stuessy and Brunken, 1979) origins have been suggested.

To establish a sound phylogenetic framework as basis for a better understanding of roles of chromosome number change and reticulate evolution in diversification of *Melampodium*, we generated and analyzed sequence data from the nuclear ITS region as well as the plastid *mat*K gene. Internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA have been frequently and successfully used for phylogenetic studies in Asteraceae (e.g., Kimball and Crawford, 2004; Samuel et al., 2006) and in tribe Heliantheae s.l., in particular (e.g., *Balsamorhiza* and *Wyethia*, Moore and Bohs, 2003;

Dahlia, Gatt et al., 2000, Saar et al., 2003; Madiinae, Baldwin and Wessa, 2000; *Montanoa*, Plovanich and Panero, 2004). Despite legitimate criticisms concerning, among others, concerted evolution, gene silencing and conversion, or their labile nature in the genome (Álvarez and Wendel, 2003), ITS is still one of the most useful phylogenetic markers in various plant groups (Nieto Feliner and Roselló, 2007). Plastid *mat*K region has also been used successfully for species-level relationships in Asteraceae (Samuel et. al., 2003, 2006), although in this family this sequence has mostly been used for phylogenetic studies at the intergeneric level and above (e.g., Bayer et al., 2000, 2002).

The current study analyzes the phylogenetic relationships among all known species of *Melampodium*. Specifically, we address the following questions: (1) What are the phylogenetic relationships among *Melampodium*, *Acanthospermum* and *Lecocarpus*, and is *Melampodium* monophyletic? (2) How well does the current taxonomic classification (Stuessy, 1972) reflect phylogenetic relationships among the species? (3) Are the chromosome base numbers predictive of evolutionary lineages? (4) Which modes of polyploidization (auto- vs. allopolyploidy) occurred, and which parental species were involved?

2. Materials and methods

2.1. Field and laboratory methods

One to several populations of all currently recognized species and varieties of *Melampodium* were collected in the United States, Mexico and Costa Rica (Table 1). *Lecocarpus* accessions used for molecular analyses were grown in the Botanical Garden of the University of Vienna, whereas *Acanthospermum* and *Melampodium moctezumum* samples were obtained from herbarium specimens (Table 1). Closely related genera (Stuessy, 1970a; Baldwin et al., 2002; Rauscher, 2002) collected in Mexico were

Table 1. Species names, localities, voucher numbers, ploidy levels (taken from H. Weiss-Schneeweiss et al., in prep.), and GenBank accession numbers of the analyzed taxa. All vouchers deposited in WU and MEXU unless otherwise indicated; Countries: A, Argentina; CR, Costa Rica; E, Ecuador; M, México; USA, United States of America. Collectors: AR, A.L. Reina; CB, C. Blöch; CR, C.A. Rebernig; CSB, Camilla Sønderberg Brok, EO, E. Ortiz B.; GF, G. Flores; HA, H. Adsersen; IC, I. Calzada; IS, I. Sánchez; JC, J. Calónico; JV, J.L.Villaseñor; JM, J.M. Morales; LA, Loran Anderson; MB, M.H.J. Barfuss; ML, M. Lenko; TD, T.R. Van Devender; TS, T.F. Stuessy.

Taxon (chromosome base number or ploidy level)	Acc. No.	Collection details, voucher numbers	GenBank accession numbers ITS	*mat*K
Melampodiinae				
Melampodium				
Sect. *Melampodium* (*x* = 10)				
Ser. *Melampodium*				
M. americanum L. (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18592.	FJ696977	FJ697080
	2	M, Colima, 2005; TS, JV, CR & IC, 18609.	FJ696978, FJ696979	FJ697081
M. diffusum Cass. (2*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18666.	FJ696975	FJ697082
	2	M, Guerrero, 2005; TS, JV, CR & IC, 18669.	FJ696976	FJ697083
M. linearilobum DC. (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18593.	FJ696983	FJ697088
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18661.	FJ696982	FJ697089
M. longipes (A.Gray) B.L.Rob. (2*x*)	1	M, Nayarit, 2005; TS, JV, CR & IC, 18619.	FJ696984	FJ697087
	2	M, Nayarit, 2005; TS, JV, CR & IC, 18621.	FJ696985	FJ697086
M. mayfieldii B.L.Turner (4*x*)	1	M, Colima, 2005; TS, JV, CR & IC, 18613.	FJ697018	FJ697112
	2	M, Jalisco, 2006; TS, JV, CB & EO, 19019.	FJ697019-FJ697021	FJ697113
M. pilosum Stuessy (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18587.	FJ696981	FJ697084
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18590.	FJ696980	FJ697085
Ser. *Leucantha*				
M. argophyllum (A.Gray ex B.L.Rob.) S.F.Blake (6*x*)	1	M, Nuevo León, 2006; TS, JV, CR & CB, 19059.	FJ697009	FJ697110
	2	M, Nuevo León, 2006; TS, JV, CR & CB, 19060.	FJ697010-FJ697013	FJ697111
M. cinereum DC. var. *cinereum* (2*x*, 4*x*)	1	USA, Texas, Frio Co, 2005; TS & CR, 18688A.	FJ697006	FJ697101
	2	USA, Texas, Zapata Co, 2005; TS & CR, 18694A.	FJ697008	FJ697102
	3	USA, Texas, Jim Hogg Co, 2005; TS & CR, 18698S.	FJ697007	FJ697103
M. cinereum DC. var. *hirtellum* Stuessy (2*x*)	1	M, Coahuila, 2006; TS, JV, CR & CB, 19057.	FJ697015	FJ697104
	2	M, Nuevo León, 2006; TS, JV, CR & CB, 19061.	FJ697014	FJ697105
M. cinereum DC. var. *ramosissimum* DC. (A.Gray) (2*x*)	1	M, Tamaulipas, 2006; TS, JV & CB, 19063.	FJ697016	FJ697106
	2	M, Tamaulipas, 2006; TS, JV & CB, 19064.	FJ697017	FJ697107
M. leucanthum Torr. & A.Gray (2*x*, 4*x*)	1	USA, Texas, Medina Co, 2005; TS & CR, 18687.	FJ697005	FJ697108
	2	USA, Arizona, Graham Co, 2006; CR & ML, 18800.	FJ697004	–
	3	USA, Arizona, Yavapai Co, 2006; CR & ML, 18808.	FJ697003	FJ697109
Ser. *Sericea*				
M. longicorne A.Gray (6*x*)	1	USA, Arizona, Pima Co, 2006; CR & MB, 18823.	FJ697000	FJ697098
	2	USA, Arizona, Pima Co, 2006; CR & MB, 18826.	FJ697001, FJ697002	FJ697099
M. nayaritense Stuessy (4*x*)	1	M, Nayarit, 2008; JV, GF & EO, 1575.	FJ696992	FJ697091
	2	M, Nayarit, 2008; JV, GF & EO, 1577.	FJ696994-FJ696996	FJ697090
	3	M, Nayarit, 2008; JV, GF & EO, 1579.	FJ696993	FJ697092
M. pringlei B.L.Rob. (6*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18637.	FJ696990, FJ696991	FJ697097
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18650.	FJ696988	FJ697094
M. sericeum Lag. (6*x*)		M, Michoacán, 2005; TS, JV, CR & IC, 18572.	FJ696986, FJ696987	FJ697093
M. strigosum Stuessy (4*x*)	1	USA, Texas, Jeff Davis Co, 2005; CR & ML, 18728.	FJ696997, FJ696998	FJ697095
	2	M, Queretaro, 2006; TS, JV & CB, 19073.	FJ696999	FJ697096

Table 1 continued

Taxon (chromosome base number or ploidy level)	Acc. No.	Collection details, voucher numbers	GenBank accession numbers ITS	*mat*K
Ser. *Cupulata*				
M. appendiculatum B.L.Rob. (2*x*)		M, Sonora, 2006; TS, JV & CB, 19046.	FJ697030	FJ697116
M. cupulatum A.Gray (2*x*)	1	M, Sinaloa, 2006; TS, JV & CB, 19044.	FJ697031	FJ697114
	2	M, Sonora, 2006; TS, JV & CB, 19048.	FJ697032	FJ697115
M. glabribracteatum Stuessy (2*x*)		M, Oaxaca, 2005; TS, JV, CR & IC, 18654.	FJ696989	FJ697100
M. moctezumum B.L.Turner		M, Sonora, 2006; TD & AR, 2007-706 (TEX).	FJ789805, FJ789806	FJ789803
M. rosei B.L.Rob. (2*x*)	1	M, Sinaloa, 2006; TS, JV, CB & EO, 19036.	FJ697025	FJ697121
	2	M, Sinaloa, 2006; TS, JV & CB, 19043.	FJ697023, FJ697024	FJ697122
	3	M, Sinaloa, 2006; TS, JV & CB, 19049.	FJ697022	–
	4	M, Sinaloa, 2006; TS, JV, CB & EO, 19025.	FJ697026	–
M. sinuatum Brandegee (2*x*)		M, Baja California, 2006; TS & JV, 19037.	FJ697029	FJ697136
M. tenellum Hook.f. & Arn. (2*x*)	1	M, Nayarit, 2006; TS, JV, CB & EO, 19020.	FJ697028	FJ697117
	2	M, Nayarit, 2006; TS, JV, CB & EO, 19023.	FJ697027	FJ697118
Ser. *Longipila*				
M. longipilum B.L.Rob. (2*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18630.	FJ696972, FJ696973	FJ697119
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18653.	FJ696974	FJ697120
Sect. *Bibractiaria* (*x* = 14)				
M. bibracteatum S.Watson (4*x*)	1	M, México, 2005; TS, JV, CR & IC, 18565.	FJ697056	FJ697145
	2	M, Durango, 2006; TS, JV, CR & CB, 19052.	FJ697057	FJ697146
M. repens Sessé & Moc. (2*x*, 4*x*)	1	M, Morelos, 2005; TS, JV, CR & IC, 18563.	FJ697059	FJ697147
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18639.	FJ697058	FJ697148
Sect. *Zarabellia* (*x* = 9)				
M. gracile Less. (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18586.	FJ697072	FJ697162
	2	M, Guerrero, 2005; TS, JV, CR & IC, 18674.	FJ697073	FJ697163
M. longifolium Cerv. ex Cav. (2*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18629.	FJ697068	FJ697142
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18633.	FJ697067	FJ697143
	3	M, México D.F., 2006; TS, JV & CB, 19074.	–	FJ697141
M. microcephalum Less. (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18569.	–	FJ697156
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18641.	–	FJ697157
	3	M, Oaxaca, 2005; TS, JV, CR & IC, 18644.	–	FJ697158
	4	M, Oaxaca, 2005; TS, JV, CR & IC, 18651.	FJ697070	FJ697161
	5	M, Oaxaca, 2005; TS, JV, CR & IC, 18658.	–	FJ697159
	6	M, Sinaloa, 2006; TS, JV, CB & EO, 19030.	FJ697071	FJ697160
M. mimulifolium B.L.Rob. (2*x*)		M, Oaxaca, 2005; TS, JV, CR & IC, 18656.	FJ697069	FJ697144
M. paniculatum Gardner (4*x*, 6*x*)	1	M, Chiapas, 2008; JV, EO & JC 1589.	FJ697065, FJ697066	–
	2	M, Chiapas, 2008; JV, EO & JC, 1591.	FJ697063	FJ697164
	3	M, Chiapas, 2008; JV, EO & JC, 1593.	FJ697064	FJ697165
	4	FL2935 (OS).	FJ697060–FJ697062	–
Sect. *Rhizomaria* (*x* = 11)				
M. aureum Brandegee (6*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18576.	FJ696970	FJ697151
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18635.	FJ696971	FJ697152
M. montanum Benth. var. *montanum* (2*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18640.	FJ696967	FJ697153
M. montanum Benth. var. *viridulum* Stuessy (2*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18646.	FJ696968	FJ697154
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18655.	FJ696969	FJ697155
Sect. *Alcina* (*x* = 11)				
M. glabrum S.Watson (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18598.	FJ697036	FJ697126
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18624.	FJ697035	FJ697125
M. nutans Stuessy (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18591.	FJ697034	FJ697124
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18664.	FJ697033	FJ697123
M. perfoliatum Stuessy (Cav.) H.B.K. (2*x*)	1	M, Jalisco, 2005; TS, JV, CR & IC, 18604.	FJ697038	FJ697149
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18652.	FJ697037	FJ697150
Sect. *Serratura* (*x* = 12)				
M. costaricense Stuessy (4*x*)	1	CR, Prov. San José, 2006; TS, JV & IS, 19076.	FJ697051	FJ697129
	2	CR, Prov. San José, 2006; TS, JV & IS, 19084.	FJ697052	FJ697130
M. dicoelocarpum B.L.Rob. (2*x*, 4*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18588.	FJ697039	FJ697134
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18595.	FJ697041–FJ697043	–
	3	M, Jalisco, 2005; TS, JV, CR & IC, 18603.	FJ697040	FJ697135
M. divaricatum (Rich. in Pers.)DC. (2*x*)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18594.	FJ697044	FJ697131
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18601.	FJ697045	–
	3	M, Oaxaca, 2005; TS, JV, CR & IC, 18668.	–	FJ697132

Table 1 continued

Taxon (chromosome base number or ploidy level)	Acc. No.	Collection details, voucher numbers	GenBank accession numbers ITS	*mat*K
	4	CR, Prov. San José, 2006; TS, JV & IS, 19086.	–	FJ697133
M. northingtonii B.L.Turner (4*x*)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18659.	FJ697054, FJ697055	FJ697139
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18660.	FJ697053	FJ697140
M. sinaloense Stuessy (4*x*)	1	M, Sinaloa, 2006; TS, JV, CB & EO, 19026.	FJ697048	FJ697127
	2	M, Sinaloa, 2006; TS, JV, CB & EO, 19027.	FJ697049, FJ697050	FJ697128
M. tepicense B.L.Rob. (2*x*)	1	M, Nayarit, 2005; TS, JV, CR & IC, 18615.	FJ697047	FJ697137
	2	M, Nayarit, 2005; TS, JV, CR & IC, 18617.	FJ697046	FJ697138
***Acanthospermum* (*x* = 11)**				
A. australe Kuntze		Rauscher, 2002.	AF465844	–
A. hispidum DC.	1	A, Jujuy, 1993; TS & JM, 12956 WU.	FJ696965	FJ789804
	2	USA, Florida; LA, 3481, KSC.	FJ696964	–
A. microcarpum B.L.Rob.		Rauscher, 2002	AF465845	–
***Lecocarpus* (*x* = 11)**				
L. lecocarpoides (B.L.Rob. & Greenm.) Cronquist & Stuessy		E, Galápagos, Osborn, 2001; CSB & HA, Lam1, DK.	–	FJ697078
L. pinnatifidus Decne.		E, Galápagos, Floreana, 2001; CSB & HA, Lam6, DK.	–	FJ697075
L. sp.		E, Galápagos; HA, s.n., DK & WU.	FJ696966	–
Outgroups				
Acmella oppositifolia (Lam.) R.K. Jansen		M, México, 2006; TS, JV, CB & EO, 19005.	–	FJ697074
Galinsoga parviflora Cav.		M, México, 2006; TS, JV, CB & EO, 19004.	FJ696962	FJ697076
Milleria quinqueflora L.		M, Jalisco, 2006; TS, JV, CB & EO, 19016.	FJ696961	FJ697077
Siegesbeckia flosculosa L'Hér.		Rauscher, 2002.	AF465888	–
Smallanthus maculatus (Cav.) H.Rob.		M, Querétaro, 2006; TS, JV & CB, 19072.	FJ696963	FJ697079
Trigonospermum melampodioides DC.		Rauscher, 2002.	AF465906	–

selected as outgroups (Table 1). Unless otherwise noted, voucher specimens are deposited in MEXU and WU (Table 1). Chromosome numbers and karyotypes of nearly all *Melampodium* accessions used in this study have been checked in root tip meristematic cells, and occasionally also in meiotic pollen mother cells in young flower buds using standard Feulgen staining (Weiss-Schneeweiss et al., 2007); chromosomal data will be published elsewhere.

Total genomic DNA was extracted from silica-dried leaf material or from herbarium specimens according to the CTAB-procedure (Doyle and Doyle, 1987) with some modifications (Tel-Zur et al., 1999). Ground plant material was washed 2–5 times with the sorbitol solution to remove polysaccharides (Tel-Zur et al., 1999). Some extracts were additionally purified with appropriate buffers of the nexttec™ Genomic DNA

Isolation Kit for Plants Maxi (β-version; nexttec, Leverkusen, Germany) according to the manufacturer's protocol.

The nuclear ITS region (partial 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, and partial 26S rRNA gene) was amplified using primers given in Table 2. The *trn*K intron including the complete *mat*K gene was amplified in one, two, three or six overlapping partitions, depending on material quality, using specific internal primers (Table 2). Polymerase chain reactions were carried out using 0.4 mM of each primer, ReddyMix PCR Master Mix (Abgene, Vienna, Austria) including 2.5 mM $MgCl_2$ with the addition of 4% dimethyl sulfoxide (DMSO) for ITS or 0.02% bovine serum albumin (BSA) for *mat*K. All PCR reactions were performed on an ABI thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) with initial 5 min at 80 °C followed by 36 cycles each of 30 s at 94 °C, 30 s at 52 °C (*mat*K) or at 60 °C (ITS), and 1–2.5 min at 72 °C (depending on the size of the amplified fragment) followed by a final elongation at 72 °C for 10 min. Amplified fragments were checked on 1% agarose gel and purified using exonuclease I (ExoI) and calf intestine alkaline phosphatase (CIAP) according to the manufacturer's protocol (Fermentas, St. Leon-Rot, Germany). The purified fragments were directly sequenced using dye terminator chemistry following the manufacturer's protocol (Applied Biosystems). The cycle sequencing reactions were performed using the same primers as for the PCR amplifications and internal primers where appropriate (Table 2). Sequencing reactions were run on a 3130xl Genetic Analyzer automated capillary sequencer (Applied Biosystems). Sequences were assembled in AutoAssembler ver. 1.4.0 (Applied Biosystems). ITS sequences of diploid accessions that showed double/multiple peaks, as well as of all polyploid accessions, were cloned using the pGEM-T Easy vector systems and JM109 competent cells (Promega, Madison, WI, USA) following manufacturer's instructions. Inserts of 6–18 positive

Table 2. Primers used for amplification and sequencing of ITS and *mat*K regions.

Primer	Primer sequence	Reference
trnK570 fwd.	5′-TCC AAA ATC AAA AGA GCG ATT GG-3′	Samuel et al., 2005
matK850 rev.	5′-TTT CCT TGA TAC CTA ACA TAA TGC ATG-3′	Gruenstaeudl et al., 2009
matK700 fwd.	5′-CAA TCT TCT CAC TTA CGA TCA ACA TC-3′	Gruenstaeudl et al., 2009
matK1710 rev.	5′-GCT TGC ATT TTT CAT TGC ACA CG-3′	Samuel et al., 2005
matK550 rev.	5′-GAC TAT CCC AAT TAT GAC ACT C-3′	Gruenstaeudl et al., 2009
matK350 fwd.	5′-ATC TTC CCT AGA AAG GAA AGG GG-3′	Gruenstaeudl et al., 2009
matK1200 rev.	5′-TAT CAG AAT CTG ATA AAT CGG CCC-3′	Gruenstaeudl et al., 2009
matK1000 fwd.	5′-CCC TTG ACT TTC TGG GTT ATC G-3′	Gruenstaeudl et al., 2009
matK1450 rev.	5′-GAA GAA ACT CTT GGA AAG GTC AAG G-3′	Gruenstaeudl et al., 2009
matK1300 fwd.	5′-CTT GTG CTA GAA CTT TAG CTC GTA AG-3′	Gruenstaeudl et al., 2009
AB101 fwd. (17SE)	5′-ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3′	Sun et al., 1994
AB102 rev. (26SE)	5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′	Sun et al., 1994
ITS3 fwd.	5′-GCA TCG ATG AAG AAC GCA GC-3′	White et al., 1990
ITS6 rev.	5′-ATG GTT CGC GGG ATT CTG CAA TTC ACA CC-3′	this study
ITS5 fwd.	5′-GGA AGT AAA AGT CGT AAC AAG G-3′	White et al., 1990

clones (depending on the ploidy level: 6 clones per diploid genome) were amplified using colony-PCR with universal M13 primers whereby recombinant colonies were added directly into the PCR mastermix and inserts amplified using reagents and conditions described in Park et al. (2007). All sequences are deposited in GenBank (Accession Nos. FJ696961–FJ697073 and FJ789805–FJ789806 for ITS; FJ697074–FJ697165 and FJ789803–FJ789804 for *mat*K; Table 1).

2.2. Alignment and phylogenetic analyses

Alignments were generated with Muscle 3.6 (Edgar, 2004) using default settings and improved by visual refinement using the program BioEdit 7.0.9.0 (Hall, 1999). The potential occurrence of pseudogenes among ITS copies was assessed via checking for the conserved angiosperm motif GGCRY–(4 to 7 N)–GYGYCAAGGAA (Liu and Schardl, 1994) in ITS1, GAATTGCAGAATCC within the 5.8S rDNA (Jobes and Thien, 1997), and the presence of the conserved (C1–C6) and variable (V1–V6) domains determined for plant ITS2 sequences (Hershkovitz and Zimmer, 1996). Sequences lacking any of these motifs were considered pseudogenes, and ITS sequencing was repeated using cloning as described above.

Nuclear and plastid sequence data were analyzed separately with indels treated as missing data or with indels coded using the modified complex indel coding (MCIC; Müller, 2006) as implemented in the program Seqstate 1.36 (Müller, 2005). As the method of indel coding used here involves a step matrix, the respective data set is not amenable to likelihood methods. Maximum parsimony analyses were performed using PAUP* 4.0b10 (Swofford, 2001) treating all characters as equally weighted. Heuristic searches included 1000 replicates of random sequence addition, tree bisection reconnection (TBR) branch swapping, and MulTrees on, but permitting no more than 10 trees to be held in each step. Trees were rooted using taxa outside Melampodiinae (Baldwin et al., 2002). Nodal support was assessed via bootstrap values (BS; Felsenstein, 1985), which were calculated using PAUP* 4.0b10 with 10,000 bootstrap replicates each with 20 random sequence addition replicates holding maximally 10 trees per replicate, SPR branch swapping, and MulTrees on.

The Bayesian analyses were conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The best-fit substitution models were determined using MrModeltest 2.2 (Nylander, 2004, program distributed by the author, Uppsala University, Uppsala). Initially, different partitioning schemes of the data set were tested, and since they all resulted in very similar topologies with comparable posterior probabilities, differences being restricted to poorly resolved and insufficiently supported regions (data not shown), the following partition scheme and substitution models were used for the final analyses: two partitions (the genic and the spacer regions of the ribosomal cistron) with K80 + Γ and GTR + Γ substitution models, and three partitions (*trn*K intron, the combined first and second codon position of the *mat*K gene, the third codon position of the *mat*K gene) with a F81 + Γ model for the first two and a GTR + Γ model for the third partition. The MCMC settings for all Bayesian analyses consisted of four runs with four chains each (three heated ones using the default heating scheme) for

5×10^6 generations sampling every 1,000th generation, using default priors and estimating all parameters during the analysis. The first 10%, which was well after the chains had reached stationarity as judged from plots of the likelihood and of all parameters and from split variances being <0.01, were discarded as burn-in. A majority rule consensus tree was constructed from the posterior set of 18,000 trees. Again, trees were rooted using non-Melampodiinae members of tribes Millerieae and Heliantheae.

The combinability of ITS and *mat*K was tested using the Incongruence Length Difference (ILD) test (Farris et al., 1994) implemented as partition-homogeneity test in PAUP* treating gaps as missing data and using 1000 partition replicates each comprising 100 random sequence addition replicates, and TBR branch swapping and keeping one tree each step. After exclusion of invariable characters, combinability was tested for (1) the whole data sets, (2) for data sets without *M. nutans*, *M. glabrum* and *M. longipilum*, which were resolved at conflicting positions in the different markers (see Section 3), and (3) data sets where additionally all polyploid taxa were excluded, as these might be of allopolyploid origin with potentially conflicting positions.

Conflicts and incongruences between topologies of both marker sets were visualized via consensus networks (Holland et al., 2004) as implemented in SplitsTree 4 (Huson and Bryant, 2006) using the default settings. In order to aid legibility, each species was reduced to one randomly chosen accession (except in cases of lack of species monophyly, where accordingly more accessions were retained), and the posterior set of each marker was thinned 360-fold resulting in 50 trees per marker and 100 trees in total.

Alternative phylogenetic hypotheses, specifically concerning the monophyly of currently recognized genera and sections, were tested in a Bayesian framework using Bayes factors (BF; Suchard et al., 2001). Marginal likelihoods (including their Monte Carlo error: Suchard et al., 2003; Redelings and Suchard, 2005) and BFs were calculated with Tracer 1.4 (available from http://evolve.zoo.ox.ac.uk/). As test statistic

we used the widely applied 2 × lnBF, considering $2 \times \ln BF_{\text{model 1 vs. model 2}} > 10$ as strong support for model 1 (Kass and Raftery, 1995).

3. Results

3.1. ITS

All sequences were checked for the presence of conserved angiosperm motifs (Liu and Schardl, 1994; Hershkovitz and Zimmer, 1996; Jobes and Thien, 1997). In cases where clones possessing those motifs were found, clones lacking any of these motifs were considered pseudogenes and excluded from further analyses. Since all cloned sequences of *M. longifolium* and *M. mimulifolium* showed an aberration (deletion) from the conserved angiosperm motif, they all were retained for the analyses. The conserved and variable domains described previously for ITS2 (Hershkovitz and Zimmer, 1996) could be identified in all obtained sequences, although slight changes to the published motifs were frequent. Eventually, the ITS data matrix included 115 samples (accessions and clones) from Melampodiinae, representing all *Melampodium* species, three species (four accessions) of *Acanthospermum*, two accessions of taxa of *Lecocarpus*, and one species each of *Galinsoga*, *Milleria*, *Siegesbeckia*, *Smallanthus* and *Trigonospermum* as outgroup. Sequences consisted of 91 bp from the 3'-end of the 18S rRNA gene, 254–261 bp ITS1, 158–159 bp 5.8S rRNA gene, 209–229 bp ITS2 and 62 bp from the 5'-end of the 26S rRNA gene. The final aligned matrix included 828 nucleotide characters (407 and 329 being variable and parsimony informative, respectively) and 24 coded indels of which 21 were parsimony informative (Table 3). Maximum parsimony analyses with gaps treated as missing data and with gaps coded as separate characters gave nearly identical tree topologies with highly similar nodal support (data not shown); therefore, only results from the second approach are presented. The heuristic search resulted in 3470 equally parsimonious trees with a length of 1348 steps (consistency index

Table 3. Sequence statistics for ITS and *mat*K. Abbreviations: GC% = GC-content in percent; MSD, Maximum Sequence Divergence; IG, ingroup taxa; Ac-Le, *Acanthospermum* and *Lecocarpus*; OG, outgroup taxa.

	Length	Var. char %	GC %	MSD IG (%)	MSD IG vs. Ac.-Le. (%)	MSD IG + Ac.-Le. vs. OG (%)
ITS1	254-261	70.40	49.00	31.30	23.20	30.40
ITS2	209-229	68.80	52.50	32.70	28.80	32.80
coding rRNA regions	250	15.40	52.70	–	–	–
***mat*K-*trn*K**	1831-1904	19.74	33.94	7.30	6.50	5.80
***mat*K gene**	1479-1530	20.63	32.34	10.80	7.00	8.80

excluding uninformative characters 0.47; retention index 0.89). The strict consensus tree is topologically very similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –8,636.03), differences being only a few insufficiently supported nodes (Fig. 1).

The clade of a paraphyletic *Acanthospermum* and a monophyletic *Lecocarpus* (clade VI, bootstrap [BS]/posterior probability [PP] 99/1.00) was nested within *Melampodium* (BS/PP 82/1.00), rendering the latter genus paraphyletic (Fig. 1). The alternative hypothesis of a monophyletic *Melampodium* is strongly rejected by 2 × lnBF of –28.64 (Table 4). Within *Melampodium*, several well-supported clades (BS/PP 96–100/1.00) can be distinguished (labeled from I to VII in Fig. 1; clade I' is not inferred from the plastid data [see below]). Their relationships to one another and to some single species clades are, however, poorly resolved and insufficiently supported. These clades only partly agree with current sectional classifications and thus with chromosome base number distribution. Clade I', which is a weakly supported sister group to the remaining ingroup taxa (BS/PP 52/0.81), consists of the sister groups *M. longipilum* of sect. *Melampodium* and the two species of sect. *Rhizomaria* (clade I). Clade II comprises *M. mimulifolium* and *M. longifolium* of sect. *Zarabellia*. The remaining species of this section (clade III) are found in a moderately supported group (BS/PP 56/1.00), which additionally includes *M. perfoliatum* of sect. *Alcina*, sect. *Bibractiaria* (clade IV), and

sect. *Serratura* (clade V), the latter two forming a poorly supported clade (BS/PP <50/0.85). The alternative hypothesis of a monophyletic sect. *Zarabellia* is strongly rejected by 2 × lnBF of –24.24 (Table 4). Clade VII is congruent with sect. *Melampodium* with the exception of *M. longipilum*, which instead belongs to clade I (the hypothesis of a monophyletic sect. *Melampodium* is strongly rejected as evidenced by 2 × lnBF of –53.98; Table 4). The three species, which form single species clades with ambiguous affinities to the other clades, are *M. glabrum*, *M. nutans* and *M. perfoliatum* (the latter with some ties to clades III–V, see above) and together constitute sect. *Alcina*, for which monophyly is strongly rejected (2 × lnBF of –57.72; Table 4). Concluding so far, nuclear ITS data supported only three of the currently recognized six sections (Stuessy, 1972) as monophyletic (sects. *Bibractiaria*, *Serratura*, *Rhizomaria*), whereas sects. *Melampodium* and *Zarabellia* are biphyletic and sect. *Alcina* is polyphyletic.

Several subclades can be distinguished within clade VII (Fig. 1). With the exception of ser. *Leucantha* (the clade comprising *M. argophyllum*, *M. cinereum* and *M. leucanthum*; BS/PP 100/1.00), none of the other series is inferred as monophyletic (the fifth series, the holotypic ser. *Longipila*, does not belong to clade VII; see above). Instead, species of series *Cupulata*, *Melampodium* and *Sericea* intermix with each other. The clade weakly suggested as sister to ser. *Leucantha* (BS/PP 63/0.83) comprises *M. mayfieldii* of ser. *Melampodium* and *M. longicorne* of ser. *Sericea* nested within ser. *Cupulata* (BS/PP 100/1.00). *Melampodium glabribracteatum* of ser. *Cupulata* is sister to a clade (BS/PP 100/1.00) of species of series *Melampodium* and *Sericea* (BS/PP 88/1.00), which themselves are grouped into two clades including members of both series (Fig. 1).

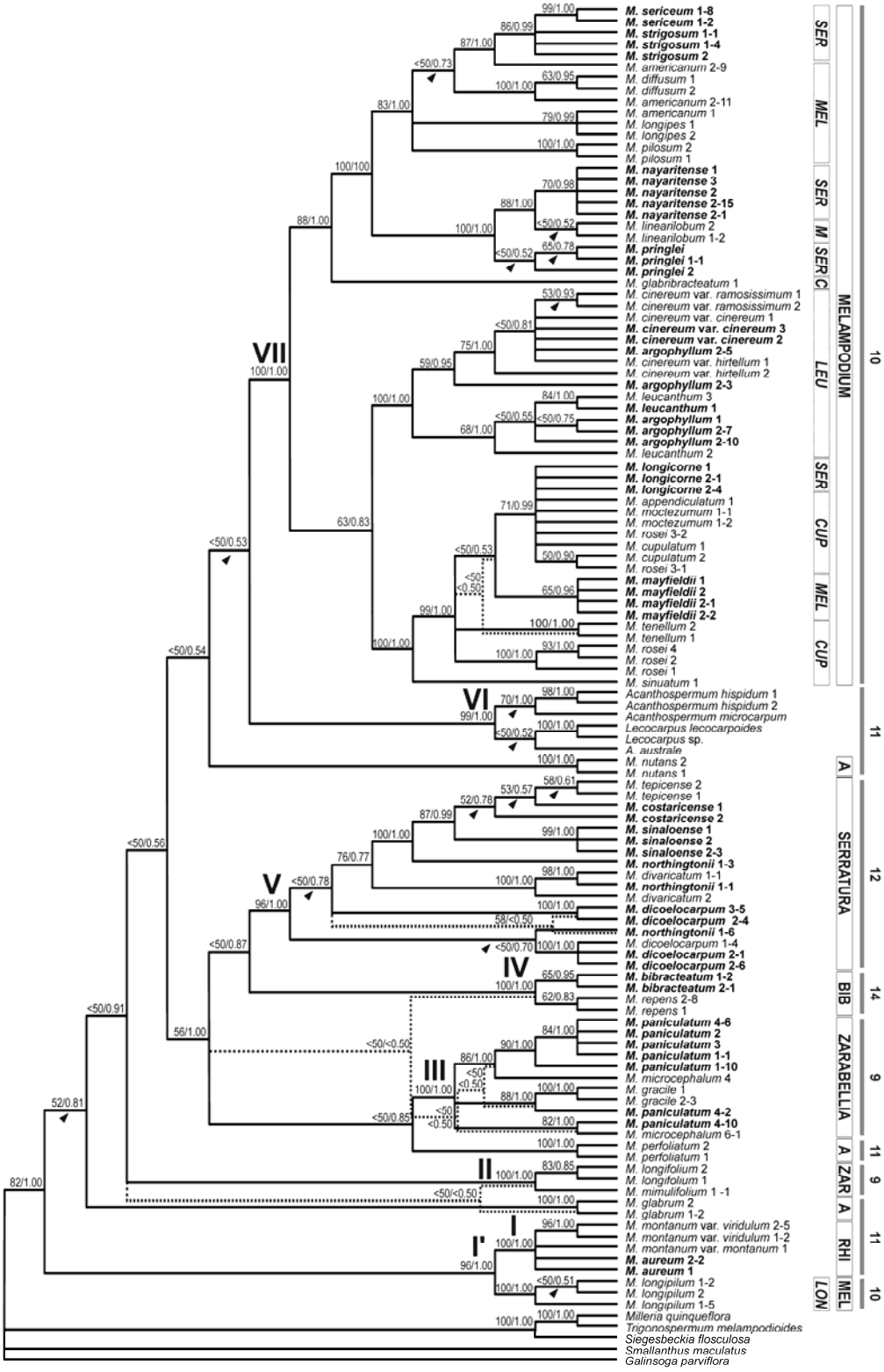

Fig. 1. Phylogenetic relationships of species of *Melampodium* and related genera inferred from Bayesian (solid lines) and maximum parsimony analysis (dotted lines) of the nuclear ITS region. Branches collapsing in the strict consensus tree are indicated by arrowheads. Numbers at nodes are bootstrap values / posterior probabilities. Numbers after species names refer to different accessions (Table 1) and to clone numbers (after dash). Polyploid taxa are indicated in bold (chromosome number of ***M. moctezumum*** not known). Clades discussed in text are indicated by Roman numerals. The basic chromosome numbers (gray bars), current sectional classification of the genus (normal font), and the series classification of sect. *Melampodium* (italics) are indicated. A, sect. *Alcina*; BIB, sect. *Bibractiaria*; MEL, sect. *Melampodium*; RHI, sect. *Rhizomaria*; ZAR, sect. *Zarabellia*; *CUP*, *C*, ser. *Cupulata*; *LEU*, ser. *Leucantha*; *L*, *LON*, ser. *Longipila*; *M*, *MEL*, ser. *Melampodium*; *SER*, ser. *Sericea*.

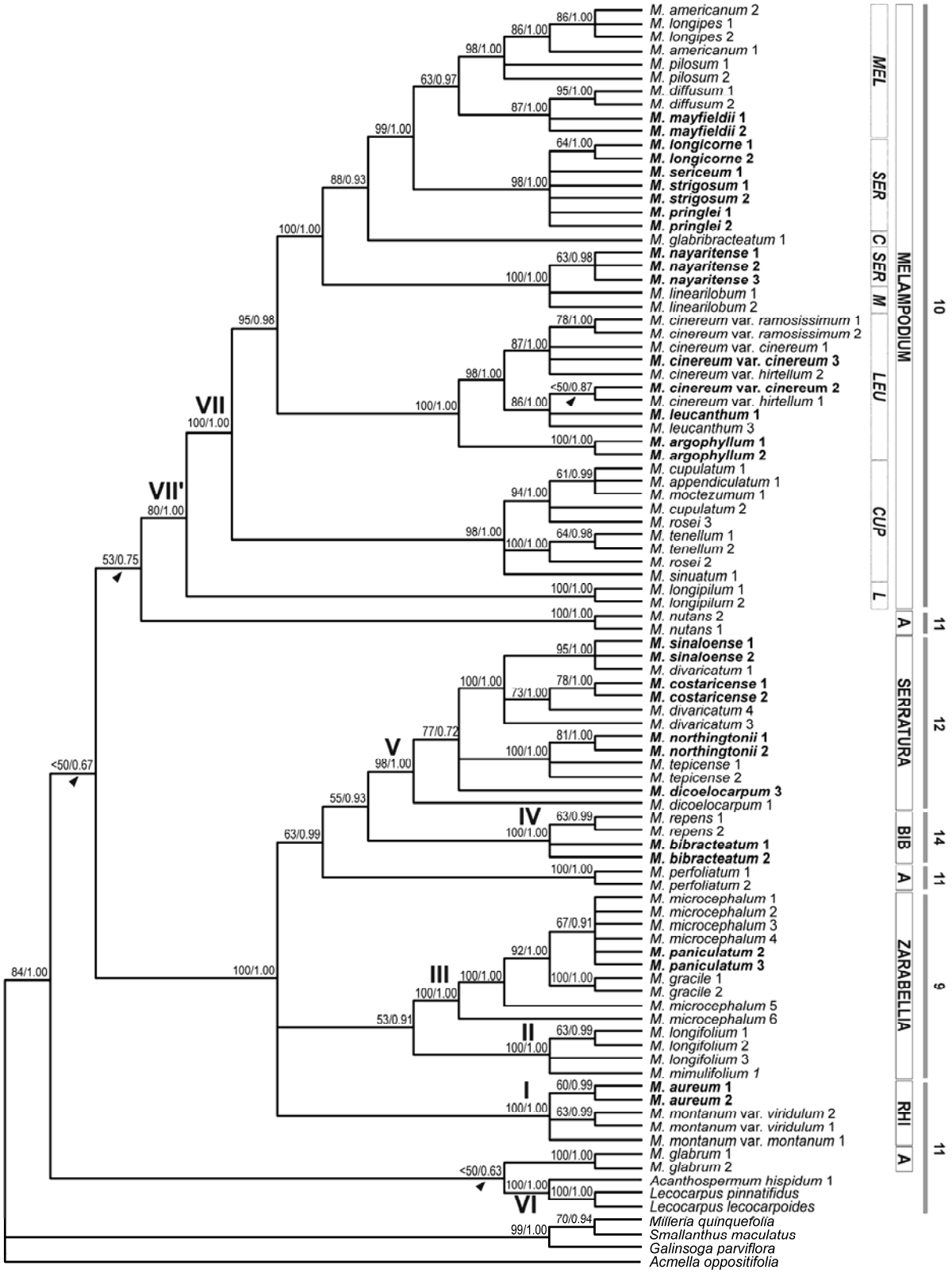

Fig. 2. Phylogenetic relationships of *Melampodium* and related genera inferred from Bayesian (solid lines) and maximum parsimony analysis (dotted lines) of the plastid *mat*K gene. Branches collapsing in the strict consensus tree are indicated by arrowheads. Numbers at nodes are bootstrap values / posterior probabilities. Numbers after species names refer to different accessions (Table 1). Polyploid taxa are indicated in bold (chromosome number of *M. moctezumum* not known). Clades discussed in text are indicated by Roman numerals. The basic chromosome numbers (gray bars), current sectional classification of the genus (normal font), and the series classification of sect. *Melampodium* (italics) are indicated (abbreviations as in Fig. 1).

*3.2. mat*K

The data matrix comprised 90 accessions of Melampodiinae and one accession each of the outgroup taxa *Acmella*, *Galinsoga*, *Milleria* and *Smallanthus* (Table 1). Sequence length was 222–394 bp for the *trn*K intron and 1479–1530 bp for the *mat*K gene, resulting in 524 and 1545 aligned characters, respectively. Of those, 427 were variable and 299 were parsimony informative. Gap coding added another seven characters for each region, adding 12 parsimonious informative characters. Again, the two different maximum parsimony analyses gave nearly identical results, with some clades being better supported in the second analysis (data not shown); again, only results from the second approach are presented. The heuristic search resulted in 9940 equally parsimonious trees with a length of 660 steps (consistency index excluding uninformative characters 0.72; retention index 0.95; Table 3). The strict consensus tree is similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –7,853.81), differences being insufficiently supported nodes (Fig. 2).

As in analyses of the nuclear data, several major clades are found (BS/PP 80–100/1.00), whose relationships among each other are unresolved or insufficiently supported (Fig. 2). To allow easier comparison with results from nuclear data, clade numbers are the same (clade VII' was not inferred from the nuclear data [see above]). Although clade VI (*Acanthospermum* and *Lecocarpus*) is nested within *Melampodium* rendering the latter genus paraphyletic, the alternative hypothesis of a monophyletic *Melampodium* cannot be rejected (2 × lnBF of –1.16; Table 4). Within *Melampodium*, clades I (sect. *Rhizomaria*), II, III (both sect. *Zarabellia*), IV (sect. *Bribractiaria*), V (sect. *Serratura*), and VII (sect. *Melampodium* except *M. longipilum*) are supported (BS/PP 98–100/1.00). Clades II and III form a weakly supported clade (BS/PP 53/0.91) as do clades IV and V (BS/PP 55/0.93), which themselves are sister to *M. perfoliatum* of sect. *Alcina* (BS/PP 63/0.99). Clades II–V plus *M. perfoliatum* together with clade I

constitute a well supported group (BS/PP 100/1.00). The phylogenetic affinities of the three species of sect. *Alcina*, for which monophyly is strongly rejected (2×lnBF of –116.60; Table 4), are, possibly with the exception of *M. perfoliatum*, unclear. Specifically, the sister-group relationship of *M. glabrum* to clade VI (BS/PP <50/0.63) and of *M. nutans* to clade VII' (clade VII plus *M. longipilum*, thus being congruent with sect. *Melampodium*; BS/PP 80/1.00) are insufficiently supported (BS/PP 53/0.75). From results of *mat*K sequence data, therefore, all currently recognized sections (Stuessy, 1972) with the exception of sect. *Alcina* are monophyletic.

Within sect. *Melampodium*, ser. *Melampodium* (except *M. linearilobum*; BS/PP 63/0.97), and ser. *Sericea* (excluding *M. nayaritense*; BS/PP 98/1.00) were found as sister groups (BS/PP 99/1.00). Subsequent sister groups are *M. glabribracteatum* of ser. *Cupulata* (BS/PP 88/0.93), a clade (BS/PP 100/1.00) of *M. linearilobum* (ser. *Melampodium*) and *M. nayaritense* (ser. *Sericea*; BS/PP 100/1.00), a well-supported (BS/PP 100.1.00) clade of ser. *Leucantha* (BS/PP 95/0.98), and the clade (BS/PP 98/1.00) of the remaining species of ser. *Cupulata* (BS/PP 100/1.00).

3.3. Incongruences between nuclear and plastid sequences

Visual inspection of phylogenetic trees derived from plastid and nuclear sequence data suggest considerable topological incongruence (Figs. 1, 2). This coincides with results from ILD tests, which reject combinability of data sets after exclusion of renegade taxa (*M. glabrum*, *M. nutans*, *M. longipilum*), and even after additional exclusion of all polyploid taxa (Figs. 1, 2; all $P = 0.001$). Instead of combining data sets, therefore, we visualize the conflicting signals in a consensus network (Fig. 3). Some of the major incongruences concern diploid taxa and clades (*M. longipilum*, clade II), whereas others involve polyploids. This is particularly pronounced in sect.

Table 4. Marginal likelihoods and their Monte Carlo error as well as the test statistic 2 x lnBF for several taxonomic hypotheses, tested separately for each marker. The compared hypotheses (unconstrained vs. alternative) are arranged in rows. 2 x $\ln BF_{\text{unconstrained vs. alternative}}$ < _10 is regarded as strong support against the alternative hypothesis.

	Unconstrained	**Monophyletic genus *Melampodium***	**Monophyletic sect. *Melampodium***	**Monophyletic sect. *Zarabellia***	**Monophyletic sect. *Alcina***
ITS					
marginal	-8,621.74 (±0.40)	-8,636.06	-8,648.73	-8,633.86	-8,650.60
likelihood		(±0.38)	(±0.37)	(±0.39)	(±0.37)
2× ln BF	–	-28.64	-53.98	-24.24	-57.72
***mat*K**					
marginal	-7,834.09 (±0.27)	-7,834.67	–	–	-7,892.39
likelihood		(±0.30)			(±0.32)
2× ln BF	–	-1.16	–	–	-116.60

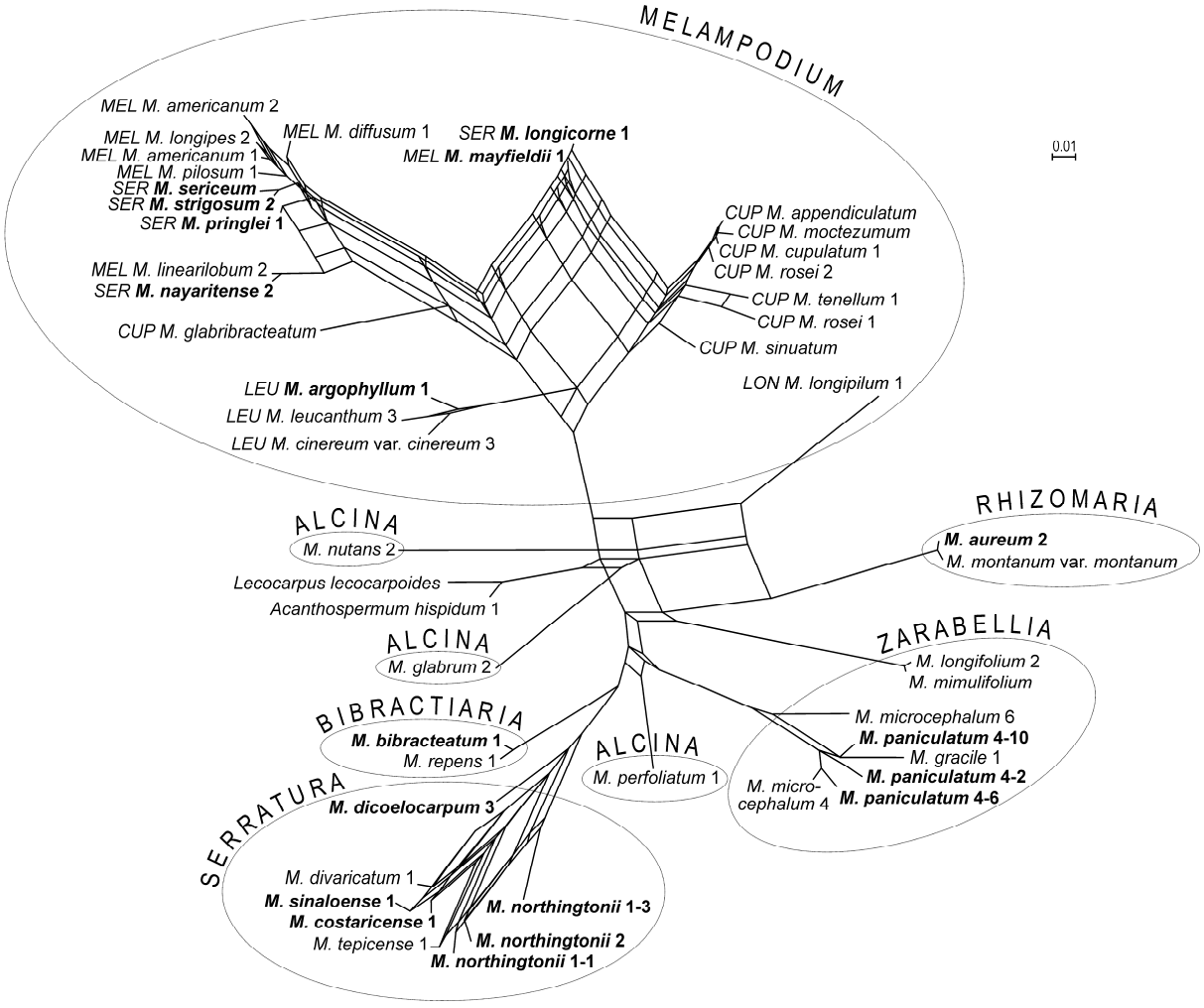

Fig. 3. Consensus network from 50 trees each of the set of posterior trees from the ITS and the *mat*K data set, respectively. Polyploid taxa are indicated in bold (chromosome number of *M. moctezumum* not known). Sectional circumscriptions (ellipses) and series memberships within sect. *Melampodium* (three-letter prefixes as in Figs. 1–2) are also shown. Scale bar represents mean edge weights.

Melampodium. The tetraploid *M. mayfieldii* (ser. *Melampodium*) and the hexaploid *M. longicorne* (ser. *Sericea*) both group with ser. *Cupulata* in the nuclear data, but instead with diploids of ser. *Melampodium* and with tetraploid *M. strigosum* of ser. *Sericea*, respectively, in the plastid data (Fig. 3). The morphologically very similar hexaploids *M. sericeum* and *M. pringlei* (both ser. *Sericea*) group with tetraploid *M. strigosum* (ser. *Sericea*) in the plastid data, but in ITS analyses only *M. sericeum* groups with *M. strigosum* whereas *M. pringlei* groups with *M. linearilobum* (ser. *Melampodium*). Conflicting positions between data sets are also seen in sect. *Serratura* (clade V), where the polyploids *M. costaricense*, *M. northingtonii* and *M. sinaloense* group with different diploids (Fig. 3).

4. Discussion

The genus *Melampodium* is a suitable system to investigate the role of chromosome number evolution (polyploidy and dysploidy) and reticulate evolution, appreciated as major forces in plant evolution and speciation (Sang et al., 1997; Rieseberg, 2001; Doyle et al., 2004; Leitch and Leitch, 2008). Assessing the role of chromosome number and reticulate evolution requires having a sound hypothesis of the phylogenetic relationships of the group (Rieseberg, 2001). Our aim here, therefore, is to establish the phylogenetic framework of *Melampodium* for further studies by testing and refining previous phylogenetic hypotheses, which were based on morphological, karyological (Stuessy, 1971, 1972, 1979; Stuessy and Brunken, 1979; Stuessy and Crisci, 1984; Stuessy et al., 2004), and phytochemical data (Seaman et al., 1980; Bohm and Stuessy, 1991).

The presence of different chromosome base numbers in *Melampodium* has been used previously to characterize infrageneric groups. Turner and King (1961) used

chromosome numbers obtained for 26 species to distinguish sect. *Melampodium* with *x* = 10 from sect. *Zarabellia* with *x* = 9, 11, 12, and 23 (*Melampodium camphoratum*, which has *x* = 16, was later excluded from the genus to *Unxia* by Stuessy, 1969). In the most recent taxonomic classification (Stuessy, 1972), four out of six sections have unique chromosome base numbers (sections *Zarabellia*, *Melampodium*, *Serratura*, and *Bibractiaria* with *x* = 9, 10, 12, and 14, respectively; Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.), and only sects. *Alcina* and *Rhizomaria* share the same chromosome base number *x* = 11 (Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.). It is obvious, therefore, that dysploidy has played an important role in the diversification of *Melampodium*.

Of the currently recognized 40 *Melampodium* species, 39 have been counted chromosomally, and 16 species contain polyploids, 13 species exclusively so (Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.), which underlines the importance of polyploid evolution in the genus. Based on morphological and karyological evidence as well as crossing experiments, some of these polyploids have been suggested to be of allopolyploid origin (Stuessy, 1971; Stuessy and Brunken, 1979; H. Weiss-Schneeweiss et al., unpubl.), emphasizing the importance of reticulate evolution for speciation within *Melampodium*.

4.1. Monophyly of Melampodium

Based on the presence of functionally staminate disk florets, pistillate ray florets and inner phyllaries each tightly enclosing and fused with single ray achenes, *Melampodium*, *Acanthospermum* and *Lecocarpus* have been grouped together in subtribe Melampodiinae (Stuessy, 1973). *Lecocarpus* differs from *Melampodium* and *Acanthospermum* by having broadly winged inner phyllaries and a shrubby habit. The latter character is often found in island groups with otherwise herbaceous relatives (e.g.,

Böhle et al., 1996; Kim et al., 1996). *Acanthospermum* differs from *Melampodium* by the presence of horn-like protuberances on the achenes. There are, however, some ambiguities concerning the morphological distinctness of *Acanthospermum*, as a similar type of achene is also found in *M. longifolium* (Stuessy, 1970a). Baillon (1882) submerged species of *Acanthospermum* and *Lecocarpus* into *Melampodium* as distinct sections, but no one has followed this suggestion. An explicit evolutionary hypothesis was put forward by Stuessy (1971), who suggested that *Acanthospermum* and *Lecocarpus* might have been derived from *Melampodium*, rendering the latter paraphyletic. Stuessy's hypothesis is supported by the nuclear sequence data (Fig. 1), which clearly reject the monophyly of *Melampodium* in favor of paraphyly ($2 \times \ln BF - 28.64$), and is at least not contradicted by the plastid data (Fig. 2 and Table 4). *Acanthospermum* and *Lecocarpus* share a chromosome base number of $x = 11$, and this base number also occurs in several phylogenetically disparate lineages of *Melampodium* (Figs. 1, 2), which suggests that it could be a plesiomorphic character for the entire group. Taxonomically, the phylogenetic position of *Acanthospermum* and *Lecocarpus* (clade VI) might be accommodated by combining both genera (corresponding to clade VI: Figs. 1, 2) or, pending the establishment of monophyly of *Acanthospermum* (but see Fig. 1), submerging them as additional two sections within *Melampodium* as suggested previously (Baillon, 1882). Alternatively, all three genera might be kept intact, following acceptance of paraphyly in classification as advocated by Stuessy (1997) and Hörandl (2007).

4.2. Phylogenetic significance of chromosome base numbers: Infrageneric relationships

Based on features of the inner phyllaries, early authors distinguished three (DeCandolle, 1836) or later two sections within genus *Melampodium* (Robinson, 1901): *Eumelampodium*, *Zarabellia*, and *Alcina* (with admittedly fewer species included). This

classification was further refined using chromosome numbers (Turner and King, 1961; Stuessy, 1971), and the current infrageneric classification (Stuessy, 1972) is fully congruent with the distribution of chromosome base numbers, suggesting a high predictive value of this character. This is, however, only partly corroborated by molecular phylogenetic data. Sect. *Bibractiaria* (clade IV) and sect. *Serratura* (clade V) both have unique base chromosome numbers ($x = 12$ and $x = 14$, respectively; Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.). Sect. *Rhizomaria* (clade I) with $x = 11$, a base number also found elsewhere in the genus, is also monophyletic (Figs. 1–3). Each section is well circumscribed morphologically. Sect. *Bibractiaria* is characterized by two outer phyllaries, sect. *Serratura* includes only annual species with five outer phyllaries with herbaceous margins, and section *Rhizomaria* includes two perennial, rhizomatous species possessing five outer phyllaries with scarious margins. A close relationship between sect. *Serratura* and sect. *Bibractiaria* has never been suggested. In both morphological phenetic and cladistic analyses sect. *Rhizomaria* has been found to tie strongly to sect. *Melampodium* (Stuessy, 1979; Stuessy and Crisci, 1984).

Contradictory evidence is found for sects. *Melampodium* and *Zarabellia*, where plastid data agree with the current taxonomy and thus distribution of chromosome base numbers (Fig. 2), but the ITS data significantly disagree (Figs. 1, 3). Of sect. *Melampodium*, plastid sequence data place *M. longipilum* (ser. *Longipila*) as sister to the remainder of the section in agreement with its chromosome base number ($x = 10$), whereas nuclear data place it instead as sister to sect. *Rhizomaria* ($x = 11$). The close relationship of *M. longipilum* and sect. *Rhizomaria* is also strongly supported by nuclear 5S rDNA intergenic spacer and low copy nuclear gene *Pgi*C sequences (C. Blöch et al., unpubl.). ITS sequences of *M. longipilum* possessed all conservative motifs, rendering the possibility of the sampled copies being pseudogenes highly unlikely. Long branch attraction artifacts are unlikely as well, because Bayesian analysis, less prone to such

difficulties, indicates relationships identical to those inferred from parsimony (Fig. 1). A unique position of *M. longipilum* within sect. *Melampodium* has already been suggested by cladistic analysis of morphological data (Stuessy, 1979). Although all species of sect. *Melampodium* share a sterile disk ovary with marked annular constriction at the point of corolla attachment, *M. longipilum* differs from the others by having an unusual flattened and apically coiled adaxial appendage on the achene, ovate subentire leaves, and markedly cupulate involucres (Stuessy, 1972). Taking the mere chromosome number as evidence for *M. longipilum* being a member of sect. *Melampodium*, its conflicting position might be the result of introgression from members of sect. *Rhizomaria* with subsequent convergence of the 35S rDNA cistron towards the introgressing genome. Alternatively, the unique karyotype of *M. longipilum*, which differs from those found in the other species of sect. *Melampodium* by a putative fusion-type chromosome pair 1 carrying an interstitial 35S rDNA locus in the pericentromeric region of the long arm (H. Weiss-Schneeweiss, unpubl.), suggests an independent origin of $x = 10$ possibly derived from $x = 11$ as found in sect. *Rhizomaria*. While further data are needed to distinguish between these hypotheses, the taxonomic consequence may be to exclude *M. longipilum* from sect. *Melampodium*. Given the likely reticulate origin of this species involving members of different sections, it might eventually be segregated into its own section.

The second case of conflicting evidence for monophyly is sect. *Zarabellia*, which is morphologically characterized by herbs with flowering heads with 3–5 outer phyllaries, often glandular. While plastid sequence data infer this section as monophyletic, albeit with weak support (Fig. 2), ITS data significantly reject this concept and point instead to two subgroups (Fig. 1 and Table 4). Although both units share the same chromosome base number of $x = 9$ as a potential synapomorphy, their karyotypes differ concerning number and localization of 5S and 35S rDNA loci (H. Weiss-Schneeweiss, unpubl.)

suggesting that x = 9 evolved twice independently. While phenetic analyses of morphological characters suggested a clear differentiation of the two groups (Stuessy and Crisci, 1984), only *M. gracile*, *M. microcephalum*, and *M. paniculatum* (clade III) form a tightly-knit evolutionary unit with all three species having only three outer phyllaries (glandular) and in which many reciprocal artificial hybridizations have been successfully performed (Stuessy and Brunken, 1979), whereas morphological synapomorphies for the morphologically disparate *M. longifolium* and *M. mimulifolium* (clade II) still remain to be found. The latter species, however, is morphologically very similar to *M. gracile* of the other subgroup (Stuessy, 1972). Further data are necessary to ascertain whether sect. *Zarabellia* is monophyletic or not and, in consequence, whether the two subclades need to be recognized as separate sections or perhaps series.

Species of sect. *Alcina* share a chromosome base number of $x = 11$, which is, however, also found in sect. *Rhizomaria* and the genera *Acanthospermum* and *Lecocarpus*, suggesting the plesiomorphic nature of this feature. The potential heterogeneity of sect. *Alcina* was already acknowledged by phenetic and cladistic analyses of morphological data (Stuessy, 1979; Stuessy and Crisci, 1984), which found *M. nutans* to be very distinct from the remainder of this section, *M. glabrum* and *M. perfoliatum*. This mostly concerns the presence of an achenial hood that is somewhat similar to those of sect. *Melampodium* plus thin stems and long petioles reminiscent of sect. *Serratura*. Plastid and nuclear ITS data now congruently suggest that sect. *Alcina* is polyphyletic (Figs. 1–3). *Melampodium perfoliatum* congruently ties in the vicinity of sects. *Bibractiaria*, *Serratura* and *Zarabellia* p.p. (clades III–V), albeit with insufficiently supported and contradictory positions (Figs. 1–2), but the phylogenetic positions of *M. glabrum* and *M. nutans* are essentially unresolved. In order to retain monophyletic groups, sect. *Alcina* in its current circumscription cannot be maintained and breaking it into three monotypic sections is one clear option.

In summary, chromosome base numbers in *Melampodium* are to a considerable extent indicative of phylogenetic relationships, as has also been found in other genera (*Hypochaeris*/Asteraceae: Cerbah et al., 1998; Samuel et al., 2003; Weiss-Schneeweiss et al., 2008; *Passiflora*/Passifloraceae: Hansen et al., 2006; *Pennisetum*/Poaceae: Martel et al., 2004; *Rhaponticum*/Asteraceae and related genera: Hidalgo et al., 2007). Despite some ambiguities and incongruences concerning the phylogenetic position of several lineages, it is obvious that chromosome base number changes (dysploidy) have played an important role in the evolution of *Melampodium*. The presence of $x = 11$ in many of the basal lineages, even if their positions are not identical in plastid and nuclear marker phylogenies, suggests $x = 11$ as the ancestral chromosome base number (maximum parsimony reconstruction, data not shown) rather than the previously hypothesized $x = 10$ (Stuessy, 1971).

4.3. Polyploidy

Polyploids are found in many groups of *Melampodium*, and one third of all *Melampodium* species are exclusively polyploid, three more also including polyploid cytotypes (Stuessy, 1970b, 1971; Stuessy et al., 2004; H. Weiss-Schneeweiss et al., unpubl.). Apart from the single species-clades of *M. glabrum*, *M. longipilum*, *M. nutans*, and *M. perfoliatum*, only clade II (*M. longifolium* and *M. mimulifolium*, sect. *Zarabellia* p.p.) and clade VI (*Acanthospermum* and *Lecocarpus*) are devoid of polyploids. Molecular phylogenetic data, in some cases strongly supported by karyological data (H. Weiss-Schneeweiss et al., unpubl.), indicate that both auto- and allopolyploidy have played significant roles in the evolution of *Melampodium*.

With increasing evidence for the frequent presence of intraspecific ploidy level variation (e.g., Weiss et al., 2003; Baack, 2004; Stuessy et al., 2004; Suda et al., 2007), recent years have witnessed appreciation of the role of autopolyploidy in speciation

(Soltis et al., 2007). Autopolyploid speciation is well supported morphologically and karyologically for sect. *Rhizomaria* (Stuessy, 1971; H. Weiss-Schneeweiss et al., unpubl.), where the hexaploid *M. aureum* is morphologically and ecologically so similar to the diploid *M. montanum* that they have been treated as a single species by McVaugh (1984). In contrast, in sect. *Bibractiaria* autopolyploidization occurred independently in its two constituent species (Figs. 1–3). In *M. repens*, a prostrate herb confined to pine-oak forests, both diploids and tetraploids are known (Keil and Stuessy, 1977; the latter reported as $2n = 54$, which probably is a miscount for $2n = 4x = 56$), while in *M. bibracteatum*, an erect, subaquatic species of open wetlands, so far only tetraploids are known (H. Weiss-Schneeweiss et al, unpubl.). In species with both diploid and polyploid cytotypes (*M. dicoelocarpum* of sect. *Serratura*, and *M. cinereum* and *M. leucanthum* of sect. *Melampodium*), the evolutionary significance of autopolyploidization is unclear. At least some of these polyploid lineages appear, however, to be genetically cohesive and separated, yet morphologically indistinguishable groups (C. Rebernig et al., unpubl.), as has been suggested for other diploid-autopolyploid complexes (Soltis et al., 2007).

Allopolyploidy is a common phenomenon in *Melampodium*. Since in allopolyploids nuclear ITS sequences may also converge towards the maternal parent (Álvarez and Wendel, 2003), the lack of incongruence between nuclear and plastid markers *per se* is no proof of an autopolyploid origin, and consequently from sequence data alone the number of allopolyploid origins might be underestimated. An excellent example is provided by the tetraploid *M. nayaritense* of sect. *Sericea*. In both nuclear and plastid sequence data it groups with the diploid *M. linearilobum* of sect. *Melampodium* (Figs. 1–3), which turns out to be the likely donor of the set of 20 small chromosomes, whereas the second parent, from which the other set of 20 larger chromosomes was obtained (H. Weiss-Schneeweiss et al., unpubl.), remains elusive. Although *M.*

linearilobum and *M. nayaritense* have been placed in different series, *Melampodium* and *Sericea* (Stuessy, 1972), respectively, a closer relationship between both species was already suggested by phenetic and cladistic analysis of morphological data (Stuessy, 1972; Stuessy and Crisci, 1984).

In clade III (sect. *Zarabellia* p.p.), the diploids *M. gracile* and *M. microcephalum* have been unambiguously shown to be involved in the origin of the tetraploid *M. paniculatum* (Stuessy and Brunken, 1979). The molecular data show that *M. microcephalum* comprises different genetic lineages (Figs. 1–3), which independently hybridized with *M. gracile* and gave rise to a thus polytopic *M. paniculatum*. Against early assertions, a polytopic origin of an allopolyploid taxon is considered the rule rather than the exception (e.g., Soltis et al., 2004; Leitch and Leitch, 2008). Similarly, in sect. *Serratura* different lineages within the diploids *M. divaricatum*, *M. tepicense*, and *M. dicoelocarpum* appear to have been involved in the origin of the polyploids *M. costaricense*, *M. sinaloense* and, very likely of polytopic origin, *M. northingtonii* (Figs. 1–2).

Numerous cases of allopolyploid speciation are also evident in sect. *Melampodium*. Of those, only the hexaploid *M. argophyllum* is found in the same series as its putative parents *M. cinereum* and *M. leucanthum* (ser. *Leucantha*; C. A. Rebernig et al., unpubl.; Figs. 1–2). Others appear to be the result of hybridization between species (or their ancestors) of different series. For instance, *M. mayfieldii* of ser. *Melampodium* nests within ser. *Cupulata* in the nuclear ITS data (Fig. 1), but groups with *M. diffusum* of ser. *Melampodium* in the *mat*K data (Fig. 2). A hotspot of allopolyploid speciation is the exclusively polyploid ser. *Sericea*. The tetraploid *M. strigosum*, itself likely of allopolyploid origin involving possibly ancestors of *M. americanum* of ser. *Melampodium* and *M. glabribracteatum* of ser. *Cupulata* (Figs. 1–3; C. Blöch et al., unpubl.), is clearly the parental taxon of the three hexaploids *M. longicorne*, *M.*

sericeum and *M. pringlei* (Figs. 1–3), with the second parental species either belonging to ser. *Cupulata* (in case of *M. longicorne*, Figs. 1–3) or being *M. linearilobum* of ser. *Melampodium* (at least in case of *M. pringlei*, Figs. 1–3), the same species also involved with the origin of the allotetraploid *M. nayaritense* (see above).

Within sect. *Melampodium*, several taxonomic series have been distinguished (Stuessy, 1972). The monotypic ser. *Longipila* might best be treated in its own section (see above). When only diploids (and their autotetraploid derivatives; Stuessy et al., 2004) are considered, ser. *Leucantha* and ser. *Melampodium* are monophyletic (except for the position of *M. linearilobum* in the ITS dataset). Once *M. glabribracteatum* has been removed from ser. *Cupulata* and transferred to its own monotypic series, ser. *Cupulata* also becomes monophyletic. When allopolyploid species are considered as well, this is, however, no longer the case. For one, several species of ser. *Sericea* nest within ser. *Melampodium* (Figs. 1–3). Species of ser. *Sericea* are very small-headed, few flowered, inconspicuous plants adapted to higher elevations, with short ray corollas, whereas those of ser. *Melampodium* are much more robust in all respects and occur in lower tropical or subtropical environments. The morphological convergence of members of ser. *Sericea*, despite their different phylogenetic origin, suggests that these characters are directly or indirectly connected with allopolyploidization. Even if ser. *Sericea* were to be merged with ser. *Melampodium*, monophyly of a thus enlarged ser. *Melampodium* is still rejected because *M. longicorne* and *M. mayfieldii* clearly connect ser. *Cupulata* with ser. *Melampodium* and ser. *Sericea* (Fig. 3). The complex pattern of a bifurcating phylogenetic structure in diploids overlain with reticulate relationships stemming from the allopolyploids has non-trivial implications for taxonomic classification. Alternatives include eliminating recognition of different series altogether or putting allopolyploids, which have parents belonging to different series, into their own series, although this might not be morphologically diagnosable. A formal re-

evaluation of current classification in the light of these new molecular data will be published elsewhere[3].

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RECONSTRUCTING BASIC CHROMOSOME NUMBER EVOLUTION IN THE GENUS *MELAMPODIUM* (ASTERACEAE)

Cordula Blöch[1], Hanna Weiss-Schneeweiss[1,] Gerald M. Schneeweiss[2] & Tod F. Stuessy[1]

[1]Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

[2]Department of Biogeography and the Botanical Garden, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

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Abstract

Melampodium is a middle-sized genus comprising a wide range of haploid chromosome numbers (*n* = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33), which can be ascribed to five basic chromosome numbers (*x* = 9, 10, 11, 12, 14). Basic chromosome numbers are in part delimiting characters for the sectional classification in *Melampodium*. This study aims to infer the ancestral chromosome number of the genus, to test whether chromosome numbers originated repeatedly, and to elucidate the directionality of the dysploid chromosome number changes. Plastid and nuclear phylogenies of the diploid species of the genus have been used for the reconstruction of ancestral chromosome number character states. While both analyzed phylogenies depict partly differing scenarios of the chromosome number evolution, both support *x* = 11 as the most likely ancestral chromosome number for the genus. Accordingly, the ancestral *x* = 11 is recovered as a symplesiomorphic character appearing in several unrelated lineages. All other chromosomal base numbers (x = 9, 10, 12, and 14) are reconstructed to be derived from *x* = 11. The chromosomal base numbers of *x* = 12 and *x* = 14 share a common ancestor most likely based on *x* = 11 and are sister to *M. perfoliatum* (*x* = 11). The previously suggested putative ancestral base chromosome number of *x* = 10 has now been shown to be derived from *x* = 11 either in a single event (reconstruction from plastid data) or from two independent events (nuclear data). Similarly, both single origin (weakly supported in plastid data) or two independent origins (supported by nuclear data) of the *x* = 9 lineage are likely. Descending dysploidy is more prevalent in *Melampodium* than ascending chromosome number change.

Keywords: basic chromosome number; character state reconstruction; ascending and descending dysploidy; chromosome number change; *Melampodium*

Introduction

Chromosomal change plays an important role in plant evolution and diversification (Stebbins 1950; White 1978; Grant 1981; Rieseberg 2001; Levin 2002; Ayala and Coluzzi 2005) creating or strengthening the barriers of interspecific gene flow (King 1993; Delneri et al. 2003). Two main types of chromosomal change impact genome evolution: numerical changes (dysploidy and polyploidy) and structural changes (karyotype rearrangements and genome size change without chromosome number change; Levin 2002; Guerra 2008). These two types of karyotypic changes are interrelated and often co-occurring. In evolutionary context dysploidy, in contrast to polyploidy and aneuploidy, does not usually lead to a dramatic change of amount of genetic material, although it changes the genomic architecture (Stebbins 1971; King 1993, Levin 2002; Lysak et al. 2006; Luo et al. 2009). Structural karyotypic changes alone involving, e.g., inversions, translocations, intra- and interchromosomal segment transposition, duplications or deletions, usually do not directly result in chromosome number change. When combined in the simplest cases, however, they may result in fusions and fissions of the Robertsonian type and change basic chromosome number. Robertsonian fission and fusion events have frequently been detected in animals (e.g., 31 centric fusions reported in *Planipillus*, Rockman and Rowell 2002), and several plant groups (e.g., *Tradescantia*, Jones 1998; *Christensonella*, Koehler et al. 2008). Simple fusion-fission events are manifested by change of number of metacentric vs. acrocentric chromosomes in closely related taxa while retaining the constant number of long chromosomal arms (Stebbins 1971; Jones 1998). In *Arabidopsis* and related genera of Brassicaceae, comparative chromosome painting allowed detection of stepwise chromosomal changes involved with evolution of different chromosomal base numbers and indicated that such changes are much more complex than simple fusion-fission events (Lysak et al. 2006; Mandáková and Lysak 2008). Additionally, dysploidy,

together with aneuploidy and other rearrangements, may act on different ploidy levels, participating in cytological and genetic diploidization of polyploids over longer evolutionary times (Lysak et al. 2006).

The major prerequisite for meaningful interpretation of directionality of dysploid change within taxa, and for inference of the ancestral basic chromosome number, is a good hypothesis on species relationships. Exhaustive information on haploid and basic chromosome numbers is also obviously needed. Such analyses have so far been largely intuitive and analyzed within the framework of morphological variation. Recent advances in molecular phylogenetic methods and their use for analyzing evolutionary relationships in plants have provided much better tools for studying the evolution of different characters, chromosome number among others.

The change of basic chromosome numbers within a group of closely related taxa may occur in different directions, resulting in descending (reduction of basic chromosome number), ascending (basic chromosome number increase) or mixed dysploid series (a combination of reduced and increased basic chromosome numbers). Descending dysploid series have been invoked to be more common in plants than ascending (Goldblatt and Johnson 1988; Goldblatt and Takei 1997). This view is now challenged by the recent analyses based on molecular phylogenetic data. Discrimination between simple ascending dysploidy and chromosome number change following genome rearrangements after paleopolyploidization is uncertain when only classical cytological methods are used.

Classic examples of dysploid series have been suggested for *Clarkia* (Onagraceae; descending dysploidy: $x = 7, 6, 5$; Lewis 1953), *Crepis* (Asteraceae; descending dysploidy: $x = 6, 5, 4, 3$; Babcock 1947a,b), *Crocus* (Iridaceae; ascending dysploidy: $x = 12, 13, 14, 15$; Brighton 1978), and *Haplopappus* (Asteraceae; descending dysploidy: $x = 9, 6, 5, 4, 3, 2$; Raven et al. 1960; Jackson 1962). However, recent molecular

phylogenetic analyses of genus *Crepis* indicated that not only is the genus polyphyletic but also the chromosome numbers are not correlated with phylogeny (Enke and Gemeinholzer 2008). This contrasts with earlier views on simple progressive evolution of basic chromosome numbers within this genus (Babcock 1947a, b). Recent molecular studies of several other plant groups have revealed chromosome numbers to be either largely uncorrelated to the phylogenetic relationships (e.g., *Artemisia*/Asteraceae: Torrell et al. 1999; Balsaminaceae: Yuan et al. 2004; *Primula*/Primulaceae: Mast et al. 2001; *Trifolium*/Fabaceae: Ellison et al. 2006; *Carex*/Cyperaceae, Hipp 2007; Hipp et al. 2009) or shown to be valuable as diagnostic characters for infrageneric classifications (e.g., *Dahlia*/Asteraceae: Gatt et al. 2000; *Hypochaeris*/Asteraceae, Cerbah et al. 1998, Samuel et al. 2003 ; Weiss-Schneeweiss et al. 2008; *Melampodium*/Asteraceae, Blöch et al. 2009; *Passiflora*/Passifloraceae, Hansen et al. 2006; *Rhaponticum*/Asteraceae and related genera, Hidalgo et al. 2007).

Chromosome number change has played an important role in the evolution of the medium-sized Asteraceae genus *Melampodium* (Asteraceae; Stuessy 1971, 1972, 1979). Basic chromosome numbers were shown to largely correlate with the sectional classification of the genus (Blöch et al. 2009; Weiss-Schneeweiss et al. 2009). A series of haploid chromosome numbers (n = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33) has been documented in *Melampodium*, and five basic chromosome numbers were inferred: x = 9, 10, 11, 12 and 14 (Stuessy 1971; Weiss-Schneeweiss et al. 2009). In the current classification of the genus (Stuessy, 1972) basic chromosome numbers in combination with morphological characters have been used to classify all species of the genus into six sections. The revised taxonomical treatment of T. F. Stuessy et al. (in prep.[4]) remains largely unchanged at the sectional level, except for the former sect. *Alcina* (x = 11) now being split into three monospecific sections (sects. *Alcina* s.str.,

[4] see Appendix p. 143.

Nutantes, and *Glabrata*). The new treatment also differs in the circumscription of some of the series in sects. *Melampodium* and *Zarabellia*. The new classification is used in the present study.

Previous intuitive analyses suggested $x = 10$ to be an ancestral basic chromosome number from which all other numbers were derived either by loss ($x = 9$) or gain ($x = 11$ and $x = 12$) of chromosomes (Stuessy 1971, 1979; $x = 14$ was not yet known). The chromosome number of $x = 10$ is found exclusively in sect. *Melampodium*, which is morphologically highly variable and species-rich (22 species). This section has a putatively primitive type of sterile disc ovary, found also in the allied genera *Acanthospermum* and *Lecocarpus* (Stuessy 1971, 1972). Sections *Zarabellia* ($x = 9$), *Serratura* ($x = 12$), and *Bibractearia* ($x = 14$; formerly erroneously assigned to $x = 9$ due to single inaccurate count of $n = 27$; L. Anderson in Keil and Stuessy 1977) have each a unique basic chromosome number. Only two sections, sects. *Rhizomaria* and *Alcina*, share common a basic chromosome number of $x = 11$ (Stuessy 1972). Except for the aforementioned sect. *Melampodium*, all remaining sections share the putatively derived sterile disc ovary type. The base chromosome number of $x = 14$ was described only recently (Weiss-Schneeweiss et al. 2009). In conflict with the hypothesis of $x = 10$ being the ancestral base chromosome number has been the presence of $x = 11$ in the closely related genera *Acanthospermum* and *Lecocarpus* (Stuessy 1971; Keil et al. 1988; Weiss-Schneeweiss et al. 2009). Recent phylogenetic analyses indicate that *Acanthospermum* and *Lecocarpus* originate from within *Melampodium* (Blöch et al. 2009), and thus, their basic chromosome number ($x = 11$) can no longer be considered as a direct "outgroup" ancestral chromosome number.

Melampodium is an excellent model group in which to analyse the evolution of basic chromosome numbers. The complete phylogeny of the genus (including all species) is now available (Blöch et al. 2009) and chromosome numbers/ploidy levels are

known for all species (Weiss-Schneeweiss et al. 2009; J. Rauchova and H. Weiss-Schneeweiss unpubl.). These data permit in-depth analyses of the chromosome number evolution in *Melampodium*. In this study maximum parsimony and maximum likelihood ancestral character state reconstructions were applied to plastid (*mat*K) and nuclear (ITS) phylogenies of diploids to infer evolution of chromosome number change. These approaches aimed to answer the following questions: (1) Which basic chromosome number is reconstructed as ancestral for the entire genus? (2) Have the different basic chromosome numbers of the genus evolved once or recurrently? and (3) Is the basic chromosome number distribution in *Melampodium* indicative of descending, ascending or mixed type of dysploidy?

Materials & Methods

Sequences of the plastid *mat*K and the nuclear ITS regions from Blöch et al. (2009) were used (Table 1). The dataset included only DNA sequences of diploid accessions to avoid confounding effects of allopolyploidy on the phylogeny. *Melampodium moctezumum* (ser. *Cupulata*, sect. *Melampodium*) was excluded from the analyses, because its exact chromosome number is not known (genome size data indicate that it is DNA-diploid; J. Rauchova and H. Weiss-Schneeweiss unpubl.). Usually, each species and each intraspecific taxon (varieties in *M. cinereum* and *M. montanum*) was represented by a single sequence. Only with high intraspecific sequence variation was more than one accession retained for analyses employing the following criterion. Briefly, inter- and intraspecific pairwise differences of the sequences using K2P distances were calculated with MEGA v. 3.1 (Tamura et al. 2007). A distance threshold was defined as the median value of interspecific distances in the region, where inter- and intraspecific distances overlapped. The median value was preferred over the

Table 1: Species names, voucher numbers, and GenBank accession numbers of the analyzed taxa. For voucher details refer to Blöch et al. (2009).

Taxon (chromosome base number)	Accession	GenBank accession numbers ITS	*mat*K
Melampodium			
Sect. *Melampodium* (*x* = 10)			
Ser. *Melampodium*			
M. americanum L.	1	FJ696977	FJ697080
	2	FJ696978, FJ696979	–
M. diffusum Cass.		FJ696975	FJ697082
M. linearilobum DC.		FJ696982	FJ697089
M. longipes (A.Gray) B.L.Rob.		FJ696985	FJ697086
M. pilosum Stuessy		FJ696980	FJ697085
Ser. *Leucantha*			
M. cinereum DC. var. *cinereum*		FJ697006	FJ697101
M. cinereum DC. var. *hirtellum* Stuessy		FJ697014	FJ697105
M. cinereum DC. var. *ramosissimum* DC. (A.Gray)		FJ697016	FJ697106
M. leucanthum Torr. & A.Gray		FJ697003	FJ697109
Ser. *Glabribracteata*			
M. glabribracteatum Stuessy		FJ696989	FJ697100
Ser. *Cupulata*			
M. appendiculatum B.L.Rob.		FJ697030	FJ697116
M. cupulatum A.Gray		FJ697031	FJ697114
M. rosei B.L.Rob.	1	FJ697023, FJ697024	FJ697122
	2	FJ697026	–
M. sinuatum Brandegee		FJ697029	FJ697136
M. tenellum Hook.f. & Arn.	1	FJ697027	FJ697118
Ser. *Longipila*			
M. longipilum B.L.Rob.		FJ696974	FJ697120
Sect. *Bibractiaria* (*x* = 14)			
M. repens Sessé & Moc.		FJ697059	FJ697147
Sect. *Zarabellia* (*x* = 9)			
Ser. *Zarabellia*			
M. longifolium Cerv. ex Cav.		FJ697068	FJ697142
M. mimulifolium B.L.Rob.	1	FJ697069	FJ697144
Ser. *Tribracteata*			
M. gracile Less.		FJ697072	FJ697162

Table 1 continued

Taxon (chromosome base number)	Accession	GenBank accession numbers ITS	*mat*K
M. microcephalum Less.	1	FJ697070	FJ697161
	2	FJ697071	FJ697160
Sect. *Rhizomaria* (*x* = 11)			
M. montanum Benth. var. *montanum*		FJ696967	FJ697153
M. montanum Benth. var. *viridulum* Stuessy	1	–	FJ697154
	2	FJ696969	–
Sect. *Glabrata* (*x* = 11)			
M. glabrum S.Watson		FJ697035	FJ697125
Sect. *Nutantes* (*x* = 11)			
M. nutans Stuessy	1	–	FJ697124
	2	FJ697033	FJ697123
Sect. *Alcina* (*x* = 11)			
M. perfoliatum Stuessy (Cav.) H.B.K.		FJ697037	FJ697150
Sect. *Serratura* (*x* = 12)			
M. dicoelocarpum B.L.Rob.	1	FJ697039	FJ697134
M. divaricatum (Rich. in Pers.) DC.	1	FJ697044	FJ697131
	2	FJ697045	–
M. tepicense B.L.Rob.		FJ697047	FJ697137
Acanthospermum (*x* = 11)			
A. australe Kuntze		AF465844	–
A. hispidum DC.		FJ696965	FJ789804
A. microcarpum B.L.Rob.		AF465845	–
Lecocarpus (*x* = 11)			
L. lecocarpoides (B.L.Rob. & Greenm.) Cronquist & Stuessy		–	FJ697078
L. pinnatifidus Decne.		–	FJ697075
L. sp.		FJ696966	–

mean in order to avoid unduly strong influence of very small interspecific distances. Intraspecific sequence data, whose pairwise distances exceeded this threshold, were kept in the dataset. The final datasets comprised 39 *Melampodium* accessions (including three each of *M. americanum* and *M. rosei*, and two each of *M. divaricatum* and *M. microcephalum*) in the ITS dataset and 34 *Melampodium* accessions (including two

each of *M. nutans* and *M. microcephalum*) in the *mat*K dataset. The trees were rooted with *Galinsoga* (x = 8, 9) and *Milleria* (x = 15) as outgroups.

Bayesian analyses were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best fit substitution models were identified using Modeltest 3.6. For ITS, the dataset was divided into the rDNA partition and the combined ITS 1 and 2 partition. Due to a high uncertainty concerning the best fit model (20 models until the cumulative Akaike weight exceeded 0.95) ranging from two to nine free parameters for the rDNA partition a moderately complex model was chosen: HKY+ Γ (5 free parameters), subsuming the proportion of invariable sites I under Gamma and modelled with six discrete categories. For the ITS 1 and 2 partition, only three models were included with eight to ten free parameters until the cumulative Akaike weight exceeded 0.95, and a GTR+ Γ was selected. For the *trn*K-intron partition of *mat*K the GTR+ Γ was selected (nine models with six to nine parameters until the cumulative Akaike weight exceeded 0.95). The same model was selected for the *mat*K-partition (four models with eight to 10 parameters until the cumulative Akaike weight exceeded 0.95). The MCMC settings for all Bayesian analyses consisted of three runs with four chains each (three heated ones using a heating parameter of 0.1 to ensure better mixing) for 25×10^6 generations sampling every 1,000th generation. The first 10% was discarded as burn-in. The combined set of 67,500 trees was thinned 15-fold resulting in a final set 4,500 trees used for all further analysis.

The ancestral states reconstruction of the chromosomal base number was calculated with the software package Mesquite v.2.7. (Maddison and Maddison 2009) using the "trace character over tree" function and displayed onto the 80 % Majority Rule Consensus tree obtained from the above described analyses. Both maximum parsimony (MP) and maximum likelihood (ML) reconstruction were employed. For the MP analysis, unordered (changes between any character state are equally costly) as well

as ordered character states (number of changes from state i to state j is |i-j|) were assumed. Both MP reconstruction modes yielded similar results, therefore only the results of the MP unordered analyses are shown in Figure 1, as these analyses implement no model. Similarly for the ML analysis the Mk1 model was employed, where the single parameter is the rate of changes between character states (all changes are equally probable). For the MP analysis, chromosome base numbers were re-coded from 0 to 6, corresponding to $x = 9$ to $x = 14$ and thus including the non-observed $x = 13$. For the ML analysis, chromosome base numbers were re-coded from 0 to 5, corresponding to the observed base numbers $x = 9, 10, 11, 12, 14$, respectively. Ancestral chromosome numbers summarized over the posterior set of 4,500 trees will be indicated as maximum likelihood reconstruction / maximum parsimony reconstruction with unordered character states / maximum parsimony reconstruction with ordered character states (ML/MP/MPord). Frequencies of ascending and descending chromosome number changes were calculated using the "summarize state changes over trees" function in Mesquite v.2.7. (Maddison and Maddison 2009) by adding all frequencies of ascending or descending chromosome number changes irrespective of the involved chromosome base number.

Results & Discussion

Ancestral base number and the $x = 11$ base chromosome number - The ancestral base number of *Melampodium* was inferred to be $x = 11$ with high support in both *mat*K and ITS dataset analyses, as well as in all ancestral character reconstruction modes (Figure 1 and Table 2; *mat*K: 1.00/1.00/0.89; ITS: 1.00/1.00/0.99). The previously suggested ancestral base number of $x = 10$ (Turner and King 1961; Stuessy 1971) was not supported in any of the analyses. The chromosomal base number of $x = 11$ occurs in several unrelated lineages in both phylogenies. The *mat*K phylogeny reveals three

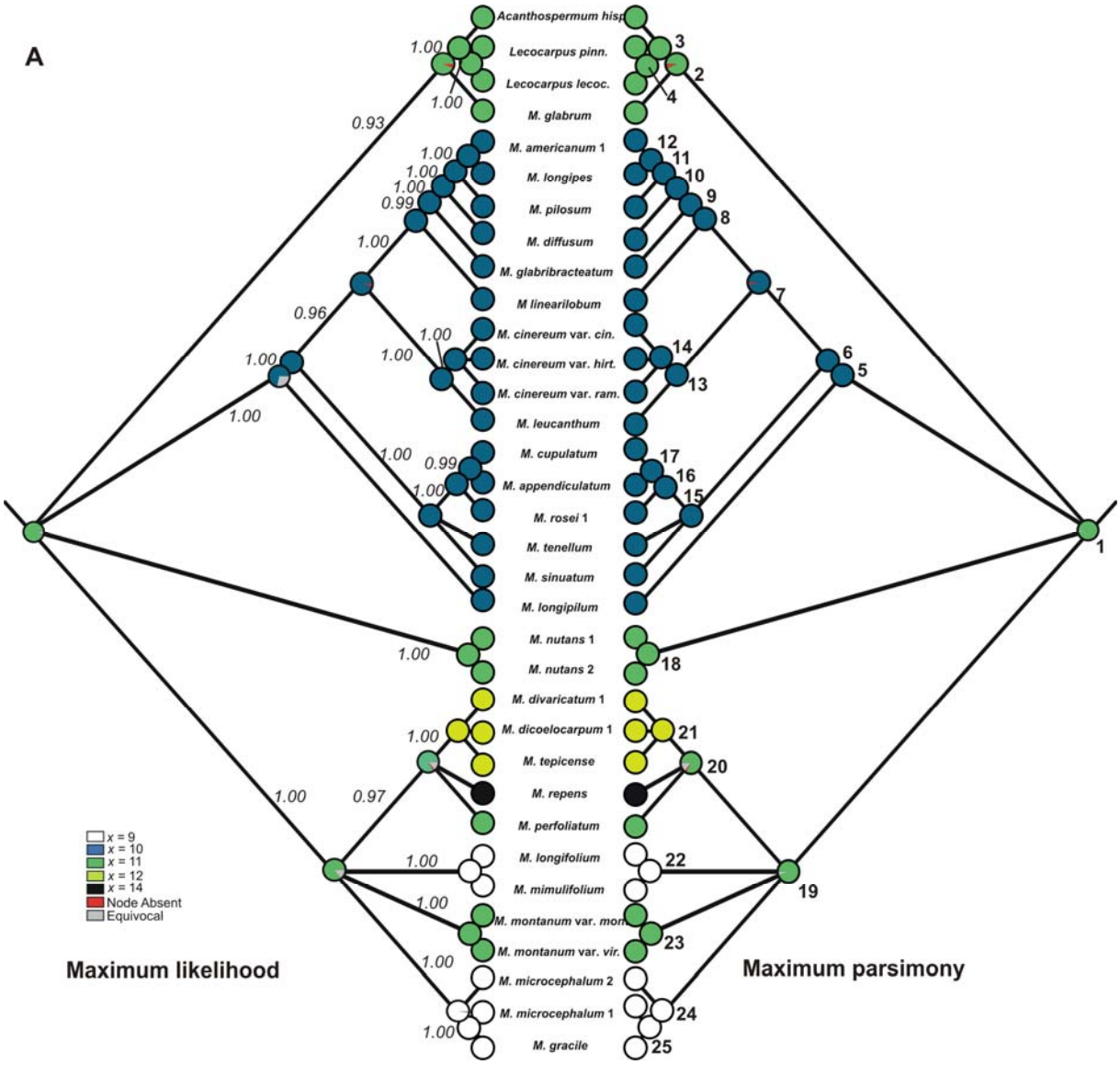

Figure 1. Ancestral character state reconstruction of basal chromosome numbers over 80% Majority Rule Consensus trees obtained by Bayesian analysis. Left: Ancestral character state reconstruction mode: maximum likelihood; Posterior probabilities for each branch are indicated in italic letters; Right: Ancestral character state reconstruction mode: maximum parsimony (with character states unordered). Nodes are numbered in bold letters (Tab. 2 gives a summary over the ancestral chromosome number reconstructions over the posterior set of 4500 trees). A: Plastid phylogeny of the *mat*K region; B: Nuclear ITS phylogeny (next page).

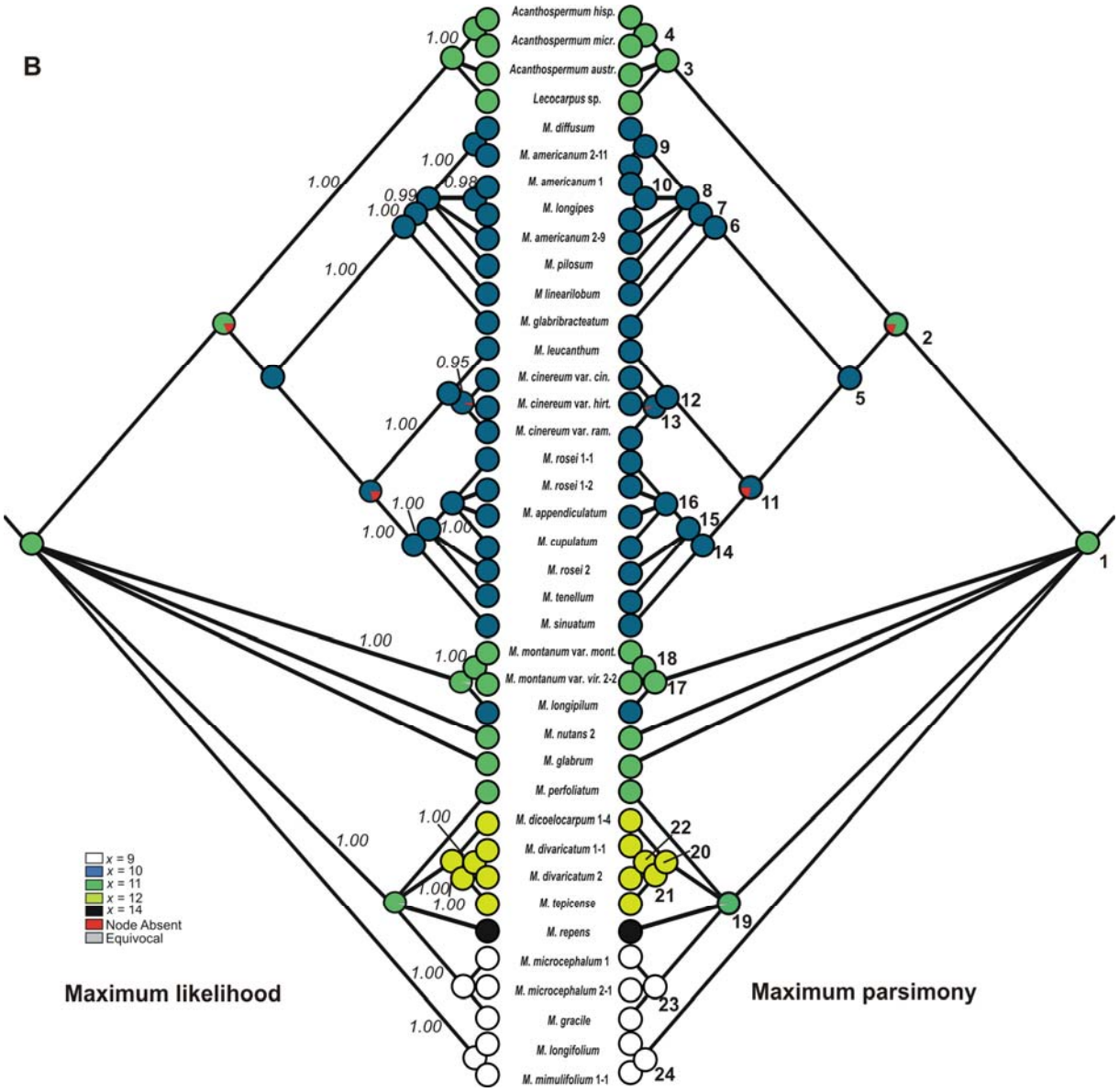
B
Acanthospermum hisp.
Acanthospermum micr.
Acanthospermum austr.
Lecocarpus sp.
M. diffusum
M. americanum 2-11
M. americanum 1
M. longipes
M. americanum 2-9
M. pilosum
M linearilobum
M. glabribracteatum
M. leucanthum
M. cinereum var. cin.
M. cinereum var. hirt.
M. cinereum var. ram.
M. rosei 1-1
M. rosei 1-2
M. appendiculatum
M. cupulatum
M. rosei 2
M. tenellum
M. sinuatum
M. montanum var. mont.
M. montanum var. vir. 2-2
M. longipilum
M. nutans 2
M. glabrum
M. perfoliatum
M. dicoelocarpum 1-4
M. divaricatum 1-1
M. divaricatum 2
M. tepicense
M. repens
M. microcephalum 1
M. microcephalum 2-1
M. gracile
M. longifolium
M. mimulifolium 1-1
1.00
0.99
0.98
0.95
x = 9
x = 10
x = 11
x = 12
x = 14
Node Absent
Equivocal
Maximum likelihood
Maximum parsimony

Table 2: Ancestral character state reconstruction on *mat*K and ITS phylogenies. Node numbers refer to node numbers marked in the right-sided trees in Fig.1. Posterior probabilities (PP) of the 4,500 trees obtained from Bayesian analyses and proportion of reconstructions of ancestral chromosome numbers over all trees are given for each node.

node	PP	ML	MP unordered	MP ordered
***mat*K**				
1		*x* = 11: 1.00	*x* = 11: 0.97	*x* = 11: 0.89
2	0.93	*x* = 11: 0.93	*x* = 11: 0.93	*x* = 11: 0.93
3	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
4	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
5	1.00	*x* = 10: 0.72	*x* = 10: 1.00	*x* = 10: 1.00
6	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
7	0.96	*x* = 10: 0.96	*x* = 10: 0.96	*x* = 10: 0.96
8	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
9	0.99	*x* = 10: 0.99	*x* = 10: 0.99	*x* = 10: 0.99
10	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
11	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
12	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
13	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
14	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
15	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
16	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
17	0.99	*x* = 10: 0.99	*x* = 10: 0.99	*x* = 10: 0.99
18	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
19	1.00	*x* = 11: 0.85	*x* = 11: 0.95	*x* = 11: 0.78
20	0.97	*x* = 11: 0.83	*x* = 11: 0.83	*x* = 11: 0.53
21	1.00	*x* = 12: 1.00	*x* = 12: 1.00	*x* = 12: 1.00
22	1.00	*x* = 9: 1.00	*x* = 9: 1.00	*x* = 9: 1.00
23	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
24	1.00	*x* = 9: 0.97	*x* = 9: 1.00	*x* = 9: 1.00
25	1.00	*x* = 9: 1.00	*x* = 9: 1.00	*x* = 9: 1.00
ITS				
1		*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 0.99
2	0.81	*x* = 11: 0.81	*x* = 11: 0.81	*x* = 11: 0.80
3	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
4	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
5	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
6	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
7	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
8	0.99	*x* = 10: 0.99	*x* = 10: 0.99	*x* = 10: 0.99
9	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
10	0.98	*x* = 10: 0.98	*x* = 10: 0.98	*x* = 10: 0.98
11	0.78	*x* = 10: 0.78	*x* = 10: 0.78	*x* = 10: 0.78
12	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
13	0.95	*x* = 10: 0.95	*x* = 10: 0.95	*x* = 10: 0.95
14	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
15	1.00	*x* = 10: 1.00	*x* = 10: 1.00	*x* = 10: 1.00
16	1.00	*x* = 10: 1.00	*x* = 10: 1.00	x = 10: 1.00
17	1.00	*x* = 11: 0.96	*x* = 11: 1.00	*x* = 11: 0.84
18	1.00	*x* = 11: 1.00	*x* = 11: 1.00	*x* = 11: 1.00
19	1.00	*x* = 11: 0.99	*x* = 11: 0.95	*x* = 11: 0.93
20	1.00	*x* = 12: 0.99	*x* = 12: 1.00	*x* = 12: 1.00
21	1.00	*x* = 12: 1.00	*x* = 12: 1.00	*x* = 12: 1.00
22	1.00	*x* = 12: 1.00	*x* = 12: 1.00	*x* = 12: 1.00
23	1.00	*x* = 9: 1.00	*x* = 9: 1.00	*x* = 9: 1.00
24	1.00	*x* = 9: 1.00	*x* = 9: 1.00	*x* = 9: 1.00

independent $x = 11$ lineages branching off of the basal polytomy: (1) *Acanthospermum* and *Lecocarpus* clade (based on $x = 11$: 1.00/1.00/1.00) with sister *M. glabrum* ($x = 11$, sect. *Glabrata* Stuessy), whose common ancestor is recovered as $x = 11$ (0.93/0.93/0.93); (2) isolated *M. nutans* ($x = 11$, sect. *Nutantes*); (3) a clade reconstructed to be derived from an $x = 11$ ancestor (0.85/0.95/0.78) and composed of sect. *Rhizomaria* ($x = 11$) and another group including species based on $x = 9$, 12, and 14 as well as *M. perfoliatum* ($x = 11$, set. *Alcina* s.str.) reconstructed to be derived from an $x = 11$ ancestor (0.83/0.83/0.53). The ITS phylogeny supports five lineages based on $x = 11$ originating from the basal polytomy: (1) *Acanthospermum* and *Lecocarpus* ($x = 11$: 1.00/1.00/1.00) as sister to sect. *Melampodium* (excluding *M. longipilum*; $x = 10$) with the ancestral node likely based on $x = 11$ (0.81/0.81/0.80); (2) the isolated *M. nutans* (sect. *Nutantes*); (3) the isolated *M. glabrum* (sect. *Glabrata*); (4) sect. *Rhizomaria* ($x = 11$: 1.00/1.00/1.00) forming clade with a *M. longipilum* with ancestral node of this whole clade based on $x = 11$ (0.96/1.00/0.84); (5) a group composed of *M. perfoliatum* of sect. *Alcina* s.str., sect. *Serratura* ($x = 12$), sect. *Bibractiaria* ($x = 14$), and sect. *Zarabellia* ser. *Tribracteata* ($x = 9$) with the ancestral node based on $x = 11$ (0.99/0.95/0.93).

The chromosome base number $x = 11$ is thus a symplesiomorphy shared by unrelated lineages. Independent origins of both sections based on $x = 11$ (sects. *Alcina* s.l. and *Rhizomaria*) were previously suggested by cladistic analyses of morphological characters (Stuessy 1979) contradicting previously suggested common ancestry of these groups (Stuessy 1971). Molecular phylogenetic analyses further suggested that the three species of sect. *Alcina* also do not share common ancestry (Blöch et al. 2009), partially agreeing with previous morphological analyses (Stuessy 1979, Stuessy and Crisci 1984). Weiss-Schneeweiss et al. (2009) did not detect any obvious karyotypic differences among species of the different $x = 11$ lineages, which would correspond to

their phylogenetic heterogeneity (*Acanthospermum* and *Lecocarpus* were not included in these analyses) and interpreted this inconspicuousness as further support for $x = 11$ being ancestral for *Melampodium*.

The $x = 9$ base chromosome number - The chromosome base of $x = 9$, unique to the species of sect. *Zarabellia*, is not supported as monophyletic in the *mat*K analyses. Instead, two lineages segregate from a polytomy (based on reconstructed ancestral $x = 11$: 0.85/0.95/0.78) as sister groups to species based on $x = 11$, 12 and 14. Both $x = 9$ lineages correspond to the two new taxonomical series recognised by T. F. Stuessy et al. (in prep.), ser. *Tribracteata* (*M. gracile* and *M. microcephalum*), and ser. *Zarabellia* (*M. mimulifolium* and *M. longifolium*). The two independent $x = 9$ lineages are suggested as even stronger in the ITS analyses. Here the species corresponding to ser. *Zarabellia* branch off the basal polytomy and the species of ser. *Tribracteata* stem out from a polytomy based on $x = 11$ (0.99/0.95/0.93), again together with species based on $x = 11$, 12 and 14.

The phenetic analyses of morphological characters (Stuessy and Crisci 1984) indicated species of sect. *Zarabellia* to be monophyletic, but divided into two groups comprising: (1) the three species of the now recently recognized ser. *Tribracteata* (T. F. Stuessy et al. in prep.), and (2) the two species of new ser. *Zarabellia* (T. F. Stuessy et al. in prep.). All five species of sect. *Zarabellia* share the chromosomal base of $x = 9$ as a potential synapomorphy, but they differ to some extent in karyotype morphology (Weiss-Schneeweiss et al. 2009), especially *M. longifolium* being different from all other species and having a more asymmetric karyotype (Weiss-Schneeweiss et al. 2009). A single origin of the two inferred $x = 9$ lineages is strongly contradicted from the ITS dataset and from previous tests on the ITS phylogeny of the entire genus using marginal likelihoods and Bayes factors (Blöch et al. 2009), but cannot be excluded from the plastid dataset (a single origin was suggested with very low support in previous

analyses; Blöch et al. 2009). The *x* = 9 karyotype could have originated once, followed by independent evolution of the two groups, but two independent origins of the *x* = 9 lineages from an *x* = 11 ancestor are equally likely.

The *x* = 10 base number - The plastid and the nuclear phylogenies are strongly incongruent concerning the origin and number of the *x* = 10 lineage(s) due to the conflicting position of one species, ***M. longipilum.*** This taxon, recovered in the plastid phylogeny (*mat*K and *psb*A-*trn*H sequences, T. F. Stuessy et al. in prep.) as sister to the rest of *x* = 10 species, groups instead in the ITS phylogeny (and in two other nuclear regions analyzed: 5S rDNA spacer and low copy *Pgi*C gene; T. F. Stuessy et al. in prep.) with sect. *Rhizomaria* (*x* = 11). Morphologically ***M. longipilum*** differs significantly from the rest of sect. *Melampodium* and was placed in the monotypic series *Longipila* of sect. *Melampodium* in the current classification (Stuessy 1972). None of the morphological analyses suggested sister relationship between ***M. longipilum*** and sect. *Rhizomaria*. Although karyotype length and genome size of ***M. longipilum*** falls into the range of the species of sect. *Melampodium*, particularly of ser. *Melampodium* (Weiss-Schneeweiss et al. 2009; B. Rupp and H. Weiss-Schneeweiss unpubl.), the karyotype of ***M. longipilum*** is distinct from all other species of the genus by possessing one interstitial locus of 35S rDNA (NOR) located in the pericentromeric region of the largest (sub)metacentric chromosome 1 (Weiss-Schneeweiss et al. 2009), instead of subterminally located NOR. This location of the NOR region may be suggestive of Robertsonian fusion within an *x* = 11 ancestral karyotype. More precise mechanisms for the origin of the other species with *x* = 10 cannot be inferred from available karyotypic data.

Thus, the plastid *mat*K region analyses suggest a single origin of the *x* = 10 lineage (with ***M. longipilum*** being basal in the *x* = 10 clade), and the nrITS analyses indicate two independent origins of *x* = 10. Marginal likelihood tests and Bayes factors

rejected monophyly of sect. *Melampodium* in the ITS dataset (Blöch et al. 2009). In the m*at*K dataset the ancestral node of the $x = 10$ lineage is reconstructed as $x = 10$ (0.72/1.00/1.00). In the Bayesian analyses of the ITS dataset one lineage of $x = 10$ taxa encompasses 21 species of the section except for *M. longipilum* ($x = 10$: 1.00/1.00/1.00), and shares a common ancestry with the genera *Lecocarpus* and *Acanthospermum* with an ancestral node most likely based on $x = 11$ (0.81/0.81/0.80). The second $x = 10$ lineage includes only *Melampodium longipilum* recovered as sister to sect. *Rhizomaria* ($x = 11$) with the ancestral node of this group also reconstructed to be $x = 11$ (0.96/1.00/1.00).

Common ancestry for the genera *Acanthospermum* and *Lecocarpus* (both $x = 11$) and sect. *Melampodium* (excl. *M. longipilum*) is suggested, albeit with low support, by ITS phylogeny and is further supported by putative synapomorphy of the sterile disc ovaries (Stuessy 1972). Phylogenetic analyses of the whole genus including polyploid taxa (Blöch et al. 2009) indicated that the position of *Acanthospermum* and *Lecocarpus* within *Melampodium* is ambiguous. In the most recent taxonomic treatment of *Melampodium*, Stuessy (1972) chose to maintain *Acanthospermum* and *Lecocarpus* at the generic level, while recognizing the origin of the two genera from within *Melampodium*.

The present analyses suggest two scenarios for the evolution of the chromosome base number of $x = 10$: (1) a single origin of the $x = 10$ lineage (supported by plastid phylogeny and karyotype length) and early lineage differentiation; (2) two independent origins of the $x = 10$ lineages (*M. longipilum* vs. the rest of $x = 10$ species), as supported by the karyotype features and all nuclear phylogenies, followed by chloroplast capture in *M. longipilum*.

The $x = 12$ and $x = 14$ base numbers – Monophyletic sections *Serratura* ($x = 12$) and *Bibracteria* ($x = 14$) were recovered in all analyses to group with one another and to

share a common ancestor of *x* = 11 (*mat*K: 0.83/0.83/0.53; ITS: 0.99/.0.95/0.93) with ***M. perfoliatum*** (*x* = 11; sect. *Alcina* s.str.). In the ITS phylogeny ser. *Tribracteata* of sect. *Zarabellia* (*x* = 9) has additionally been included in this group.

The relationship of sect. *Serratura* to sect. *Bibractearia* and to ***M. perfoliatum*** has already been recovered in cladistic analysis of morphological characters but was interpreted as rather unlikely (Stuessy 1979). Karyotypically, no particular features tie ***M. perfoliatum*** to any of the species of *x* = 12 and *x* = 14 lineages (Weiss-Schneeweiss et al. 2009), except for perhaps slightly larger size of the chromosomes. Karyotype analyses did not allow speculations on the mechanisms leading to the origin of the two unique chromosome numbers (*x* = 12, 14).

Chromosome number changes in *Melampodium* – *Melampodium* displays one of the longest chains of both basic and haploid chromosome numbers in the family Asteraceae. The diversity of chromosome numbers in *Melampodium* results from dysploidy, frequent polyploidization, and polyploidy-associated dysploidy/aneuploidy. Basic chromosome number evolution in *Melampodium* can be attributed to both descending and ascending dysploidy (Figure 1, Table 2). Descending dysploidy is suggested for two lineages according to the plastid *mat*K analyses and for four lineages according to the nuclear ITS analyses (average number of chromosome number reduction events over all trees: *mat*K analyses: 1.37/2.60/2.66; ITS analyses: 3.07/4.03/4.04). Ascending dysploidy occurred in both phylogenies on one branch leading to *x* = 12 and *x* = 14 (average number of events leading to chromosome number increase over all trees: *mat*K: 0.77/1.90/2.29; ITS: 0.96/1.97/2.10).

These results partially support previous cladistic analyses of morphological characters (Stuessy 1979), which assumed a mixed type of ascending and descending dysploidy, but proposed *x* = 10 as the ancestral chromosome number. Accordingly, when *x* = 10 has been assumed to be the ancestral chromosome number, ascending

dysploidy prevailed with the only dysploid loss restricted to change from $x = 10$ to $x = 9$.

Hypotheses of chromosome number evolution suggested by morphological or cytological data have recently been tested using DNA sequence data in several other plant groups. While some of these analyses strongly contradicted previous hypotheses (e.g., *Crepis*/Asteraceae, Babcock 1947a,b, Enke and Gemeinholzer 2008; *Pennisetum*/Poaceae, Rao et al. 1989, Martel et al. 2004), others provided strong support (e.g., *Pelargonium*/Geraniaceae, Bakker et al. 2000). Among numerous plant groups studied to date, both descending (*Brachyscome*/Asteraceae, Watanabe et al. 1999; *Christensonella*/Orchidaceae, Koehler et al. 2008; *Podolepis*/Asteraceae, Konishi et al. 2000), and more rarely ascending dysploidy (*Borago*/Boraginaceae, Selvi 2006; *Crepis*/Asteraceae, Enke and Gemeinholzer 2008) have been suggested. Both types have occasionally also been hypothesized to originate recurrently (*Brachyscome*/Asteraceae, Watanabe et al. 1999; *Crepis*/Asteraceae, Enke and Gemeinholzer 2008). A mixed type of dysploidy (involving both ascending and descending) has been suggested for some plant genera (e.g., *Clarkia/*Onograceae, Lewis 1953, Gottlieb and Ford 1996; *Hypochaeris*/Asteraceae, Cerbah et al. 1998; Samuel et al. 2003; *Pelargonium*/Geraniaceae, Bakker et al. 2000).

Although existing data do not allow definite statements about prevalence of either descending or ascending dysploidy in plants, the former has so far been inferred more often (Goldblatt and Johnson, 1988; Goldblatt and Takei 1997). Convincing theories for the prevalence of one type of dysploid change over the other are lacking. Descending dysploidy may be more common due to widespread polyploidization in flowering plants. Polyploidy creates high redundancy of genetic material. Increasing the number of chromosome sets and subsequent genome diploidization have been shown in some

groups clearly to facilitate rearrangements leading to reduction of chromosome number (also loss of redundant genetic material loss; Lysak et al. 2006)

Chromosome number change in *Melampodium* has very likely acted as a barrier to gene flow, promoting lineage differentiation. Species of *Melampodium* occurring sympatrically or parapatrically usually possess different chromosome numbers (Sundberg and Stuessy 1990). Chromosomal rearrangements in combination with mating barriers are known to accelerate genic diversification between populations and facilitate speciation by, e.g., impeding gene exchange which may create/increase mating barriers and finally lead to speciation (Ayala and Coluzzi 2005) or alternatively speciation may occur aforehand and be followed by subsequent chromosomal change (Rieseberg 2001).

It is apparent that not all basic chromosome number changes in *Melampodium* have yielded equally successful genetic combinations. While most of the lineages based on $x = 9$, 11, 12, or 14 each comprise only a few species, dysploid change leading to $x = 10$ has significantly increased the rate of species diversification and speciation in *Melampodium*, contributing more than 50% of the species in the genus.

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REPEATED CYCLES OF HYBRIDIZATION AND POLYPLOIDIZATION IN *MELAMPODIUM*: ORIGIN AND GENOME EVOLUTION OF ALLOPOLYPLOIDS OF SECT. *MELAMPODIUM* (ASTERACEAE)

Cordula Blöch[1], Hanna Weiss-Schneeweiss[1], Gerald M. Schneeweiss[2], Barbara Rupp[1], José L. Villaseñor[3] & Tod F. Stuessy[1]

[1]Department of Systematic and Evolutionary Botany, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

[2]Department of Biogeography and the Botanical Garden, University of Vienna, Rennweg 14, A-1030 Vienna, Austria;

[3]Departamento de Botánica, Instituto de Biologia, Universidad Nacional Autónoma de México, México, D.F., Mexico

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Abstract

- The genus *Melampodium* is chromosomally diverse with five base chromosome numbers known (x = 9, 10, 11, 12, 14). The current study has been designed to infer the origin and analyze the genomic evolution of all polyploids of ser. *Sericea* and one of ser. *Melampodium* (all sect. *Melampodium*).
- The allopolyploid origin of tetraploid and hexaploid taxa has been inferred from analyses of several plastid (*mat*K and *psb*A-*trn*H) and nuclear (ITS, 5S rDNA, low copy *Pgi*C gene) DNA regions as well as ITS restriction patterns. 35S and 5S rDNA loci localizations within chromosomes and genome size measurements were used to investigate the dynamics of genome evolution in polyploids in comparison to diploid relatives.
- All polyploids originated via hybridization involving putative parental taxa from sers. *Melampodium, Cupulata* s.str., *Glabribracteata* and *Sericea* (all sect. *Melampodium*). Species within the series represent unique combinations of karyotypic features, patterns of rDNA loci number and localization in the chromosomes, and genome size values, except for the distinct diploid *M. linearilobum* and polyploids of ser. *Sericea*. The genome size additivity observed in all polyploids contrasts with common 35S rDNA loci loss and conversion and to a much lesser extent with loss of a few 5S rDNA loci.
- Two allohexaploid species, *M. pringlei* and *M. sericeum*, although sharing the same set of parental taxa, allotetraploid *M. strigosum* and diploid *M. linearilobum*, have undergone speciation accompanied by different genomic restructuring as judged by rDNA loci dynamics.

Keywords: allopolyploidy, chromosome evolution, FISH, genome size, hybridization, low copy nuclear gene, *Melampodium*, plastid phylogeny, rDNA.

Introduction

Hybridization and polyploidization (Whole Genome Duplication; WGD) have been conspicuous during eukaryotic evolution, and their frequency suggests they may confer selective advantage (Stebbins, 1971; Grant, 1981; Arnold, 1997; Wendel, 2000; Rieseberg, 2001; Levin, 2002; Comai, 2005; Hufton & Panopoulou, 2009). Both of these processes have been shown to be abundant in angiosperms (Stebbins, 1971; Mallet, 2007; Rieseberg & Willis, 2007) either acting alone, i.e., resulting in autopolyploids or homoploid hybrids, respectively, or in concert producing allopolyploids, i.e., hybrids with fully duplicated genomes.

Although estimates vary of the frequency of polyploid speciation in angiosperm evolution (Grant, 1981; Masterson, 1994; Otto & Whitton, 2000; Wood *et al.*, 2009), it is commonly agreed that allopolyploidy constitutes an important factor in plant speciation. Allopolyploids are frequently immediately reproductively isolated from parental lineages (Rieseberg & Willis, 2007) and are considered to undergo "instant" speciation (Otto & Whitton, 2000; Coyne & Orr, 2004; Linder & Rieseberg, 2004; Mallet, 2007; Slotte *et al.*, 2008; Ainouche *et al.*, 2009). The most common mechanisms of allopolyploid formation involve fusion of unreduced gametes (with or without triploid intermediate), and chromosome doubling of homoploid diploid hybrids (deWet, 1980; Grant, 1981; Ramsey & Schemske, 1998; DE Soltis *et al.*, 2004).

To elucidate the origin of polyploids is not an easy task. Morphological intermediacy and incongruence between gene trees obtained from chloroplast DNA (cpDNA; usually inherited maternally in angiosperms) vs. nuclear DNA (inherited from both parents; Sang *et al.*, 1995; Hughes *et al.*, 2002; Kim *et al.*, 2008) are usually the first evidence of hybridization. However, the often low variation in cpDNA sequences at the intraspecific level, and concerted evolution of the commonly used nuclear ribosomal internal transcribed spacer (nrITS) region (Álvarez & Wendel, 2003), have

limited the precision with which allopolyploidy can be identified. Recent use of low-copy nuclear genes has proven more successful in recovering individual gene copies in allopolyploids that have been contributed by maternal and paternal lineages (Sang, 2002; Small *et al.*, 2004; Lihová *et al.*, 2006; Fortune *et al.*, 2007; Kim *et al.*, 2008).

Evolution of newly formed hybrids is often accompanied by genic, genomic and epigenetic changes (Chen, 2007) prompted by genomic shock (McClintock, 1984) imposed by merging and/or duplicating two parental genomes in one cell (Comai *et al.*, 2003). Polyploidy simultaneously generates a large amount of redundant genetic material, which, when exploited, can lead to functional novelty and promote speciation (Hufton & Panopoulou, 2009; Le Comber *et al.*, 2010). Some of the allopolyploid genomes exhibit astounding ability for rapid genome rearrangements. The type and extent of such rearrangements have been shown to vary among different plant groups targeting genic, genomic, epigenetic, and chromosomal levels in various combinations (e.g., Shaked *et al.*, 2001; Salmon *et al.*, 2005; Tate *et al.*, 2006; JJ Doyle *et al.*, 2008; Lim *et al.*, 2008; AR Leitch & IJ Leitch, 2008). Those changes may occur in only a few generations (Song *et al.*, 1995; Zwierzykowski *et al.*, 1998; Hanson *et al.*, 2000; Wendel, 2000; Gaeta *et al.*, 2007; Lim *et al.*, 2008) involving, e.g., homeologous recombination (e.g., *Brassica*; Song *et al.*, 1995; Gaeta *et al.*, 2007) or rapid and reproducible elimination of non-coding DNA associated with changes in epigenetic regulation (e.g., wheat, Levy & Feldman, 2004; Bento *et al.*, 2008).

The genus *Melampodium* (Asteraceae) contains 40 species divided into six taxonomic sections (Stuessy, 1972) that are distributed in Mexico, Central America, and adjacent states of the USA. It exhibits a remarkable range of basic chromosome numbers with $x = 9$, 10, 11, 12, and 14. Polyploidy occurs in 40% of the species (both $4x$ and $6x$), with 13 species being exclusively polyploid (32%; Weiss-Schneeweiss *et al.*, 2009). Our recent molecular phylogenetic analyses of the whole genus suggested

numerous cases of allopolyploid speciation as inferred by incongruence between plastid and nuclear (ITS) gene trees (Blöch *et al.*, 2009). The largest section *Melampodium* contains five series, one of which (series *Sericea*) includes five exclusively polyploid species, two tetraploids (*M. nayaritense* and *M. strigosum*) and three hexaploids (*M. longicorne*, *M. pringlei*, and *M. sericeum*). The combination of phylogenetic and chromosomal data suggested that all of these species as well as the only tetraploid of related ser. *Melampodium*, *M. mayfieldii*, originated via hybridization involving several diploid taxa of three other related series, sers. *Cupulata* s.str, *Melampodium*, and *Glabribracteata*, accompanied by genome doubling (Blöch *et al.*, 2009; Weiss-Schneeweiss *et al.*, 2009). Multiple rounds of hybridization have also been suggested involving allotetraploid *M. strigosum*. Polyploid cytotypes of species of ser. *Leucantha* of sect. *Melampodium* were shown to be rather of autopolyploid origin (Rebernig *et al.*, 2010).

In this study we focus on the origin and genome evolution of a group of six polyploid species of sers. *Melampodium* and *Sericea* (both in sect. *Melampodium*) for which karyological evidence and/or recently published phylogenies suggested allopolyploid origin (Stuessy, 1970; Blöch *et al.*, 2009; Weiss-Schneeweiss *et al.*, 2009). To test previous hypotheses we have analyzed the sequences of plastid *mat*K and *psb*A-*trn*H regions, as well as nuclear 5S rDNA non-transcribed spacer (NTS), ITS, and two paralogues of the low copy nuclear gene *Pgi*C. Both ribosomal loci (5S and 35S rDNA) were employed as chromosomal markers for the analysis of karyotype evolution. The analyses were implemented by restriction digestion analyses of the ITS1 and ITS2 regions, as well as genome size estimations.

The specific aims of this study are to: (1) analyse origins of the six polyploid species, and infer their parental taxa; (2) assess the type and extent of genomic changes that have accompanied hybridization and polyploidization at the genomic (genome

size), chromosomal (karyotype, rDNA loci), and sequence levels; (3) analyze parallels or lack thereof in genomic evolution in two closely related allopolyploid taxa, *M. sericeum* and *M. pringlei*, that share the same parentage; and (4) infer the role of reticulate evolution in speciation within sect. *Melampodium*.

Materials & Methods

Plant material - One to several populations of all currently recognized species and varieties of *Melampodium* sect. *Melampodium* sers. *Melampodium*, *Cupulata* s.str., *Glabribracteata* and *Sericea* were collected in Mexico and the United States during several field trips in 2005-2008 (Table 1). Voucher specimens are deposited in WU and UNAM unless otherwise indicated in Table 1. In this study the new taxonomic treatment of *Melampodium* according to TF Stuessy *et al*. (in prep.; see Appendix p. 143) is applied.

DNA extraction - DNA extraction has been carried out following standard procedure (Tel-Zur *et al.*, 1999) with the modification described in Blöch *et al.* (2009).

Amplification and sequencing of nuclear and plastid markers - Amplification, cloning and sequencing of the nuclear ITS and plastid *mat*K regions followed protocols described in Blöch *et al.* (2009). The plastid *psb*A-*trn*H region was amplified as described in Rebernig *et al.* (2010) using primers of Sang *et al.* (1997).

The 5S rDNA repeat region including the NTS was amplified using newly designed primers situated in the 5S rRNA gene (forward 5'-GGTGCGATCATACCA-GCAC-3'; reverse 5'-GGTGCAACACTAGGACTTC-3'; MWG, Ebersberg, Germany). PCR was carried out with 0.5µM of each primer, 1× Ready Mix PCR Master Mix (containing 2.5 mM $MgCl_2$; Sigma, Vienna, Austria), 4% dimethyl sulphoxide (DMSO), and c. 50 ng of DNA. The cycling conditions included

Table 1. Species names, localities, voucher numbers, ploidy levels, and GenBank accession numbers of the analyzed taxa. Accessions used for ITS restriction digestion (ITS RE), FISH and genome size measurements analyses are indicated (samples marked by *: FISH data not shown in Fig. 4). All vouchers deposited in WU and UNAM unless otherwise indicated; Abbreviations: CR, Costa Rica; E, Ecuador; M, México; USA, United States of America; Collectors: CB, C. Blöch; CR, C.A. Rebernig; CSB, Camilla Sønderberg Brok; EO, E. Ortiz B.; HA, H. Adsersen; IC, I. Calzada; IS, I. Sanchez; JV, J.L.Villaseñor; MB, M.H.J. Barfuss; ML, M. Lenko; JC, J. Calónico; TS, T.F. Stuessy.

Taxon	Ploidy level	Collection details, voucher numbers	Genome size	FISH	ITS RE	GenBank accession numbers					
						ITS	*mat*K	*psb*A-*trn*H	5S rDNA	*Pgi*CI	*Pgi*CII
Ser. *Melampodium*											
M. americanum L.	2*x*	M, Michoacán, 2005; TS, JV, CR & IC, 18592.				FJ696977	FJ697080	GU216556			
M. americanum L.	2*x*	M, Colima, 2005; TS, JV, CR & IC, 18609.		✓	✓	FJ696978, FJ696979	FJ697081	GU216557	GU216359-GU216369	GU216452, GU216453	GU216547, GU216551, GU216552
M. americanum L.	2*x*	M, Michoacán, 2006; TS, JV, CB, & EO, 19009.	✓	✓							
M. americanum L.	2*x*	M, Michoacán, 2005; TS, JV, CR, & IC, 18583.	✓								
M. diffusum Cass.	2*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18666.		✓	✓	FJ696975	FJ697082	GU216554	GU216402-GU216405	GU216450, GU216451	GU216545, GU216546
M. diffusum Cass.	2*x*	M, Guerrero, 2005; TS, JV, CR & IC, 18669.	✓			FJ696976	FJ697083	GU216555			
M. diffusum Cass.	2*x*	M, Guerrero, 2005; TS, JV, CR, & IC, 18671.	✓	✓							
M. linearilobum DC.	2*x*	M, Michoacán, 2005; TS, JV, CR & IC, 18593.				FJ696983	FJ697088	GU216574	GU216428-GU216430, GU216432, GU216433	GU216442, GU216443, GU216469-GU216471	
M. linearilobum DC.	2*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18661.			✓	FJ696982	FJ697089	GU216575			

Table 1. continued

Taxon	Ploidy level	Collection details, voucher numbers	Genome size	FISH	ITS RE	GenBank accession numbers					
						ITS	*mat*K	*psb*A-*trn*H	5S rDNA	*Pgi*CI	*Pgi*CII
M. linearilobum DC.	2*x*	M, Colima, 2005; TS, JV, CR & IC, 18610									GU216531, GU216553
M. linearilobum DC.	2*x*	M, Oaxaca, 2005; TS, JV, CR, & IC, 18667.			✓						
M. linearilobum DC.	2*x*	M, Oaxaca, 2005; TS, JV, CR, & IC, 18662.	✓		✓						
M. linearilobum DC.	2*x*	M, Oaxaca, 2005; TS, JV, CR, & IC, 18665.		✓							
M. linearilobum DC.	2*x*	M, Oaxaca 2004; EO, MEXU 333.		✓							
M. longipes (A.Gray) B.L.Rob.	2*x*	M, Nayarit, 2005; TS, JV, CR & IC, 18619.			✓	FJ696984	FJ697087				
M. longipes (A.Gray) B.L.Rob.	2*x*	M, Nayarit, 2005; TS, JV, CR & IC, 18621.	✓	* ✓		FJ696985	FJ697086	GU216560	GU216406-GU216410	GU216444-GU216446	GU216543, GU216544
M. longipes (A.Gray) B.L.Rob.	2*x*	M, Jalisco, 2006;TS, JV, CB & EO, 19015	✓					GU216561			
M. mayfieldii B.L.Turner	4*x*	M, Colima, 2005; TS, JV, CR & IC, 18613.				FJ697018	FJ697087	GU216563			
M. mayfieldii B.L.Turner	4*x*	M, Jalisco, 2006; TS, JV, CB & EO, 19019.	✓	✓	✓	FJ697019, FJ697020, FJ697021	FJ697086	GU216564	GU216325-GU216330	GU216434-GU216441	GU216535-GU216542
M. pilosum Stuessy	2*x*	M, Michoacán, 2005; TS, JV, CR & IC, 18587.	✓	✓	✓	FJ696981	FJ697084	GU216558	GU216344, GU216345	GU216447-GU216449	GU216550
M. pilosum Stuessy	2*x*	M, Michoacán, 2005; TS, JV, CR & IC, 18590.				FJ696980	FJ697085	GU216559			
M. pilosum Stuessy	2*x*	M, Michoacán, 2006; TS, JV, CB, & EO, 19010.	✓								

Table 1. continued

Taxon	Ploidy level	Collection details, voucher numbers	Genome size	FISH	ITS RE	GenBank accession numbers					
						ITS	*mat*K	*psb*A-*trn*H	5S rDNA	*Pgi*CI	*Pgi*CII
Ser. *Sericea*											
M. longicorne A.Gray	6*x*	USA, Arizona, Pina Co, 2006; CR & MB, 18823.		✓		FJ697000	FJ697098	GU216570	GU216347-GU216354	GU216454, GU216455	GU216505, GU216512
M. longicorne A.Gray	6*x*	USA, Arizona, Pina Co, 2006; CR & MB, 18824.	✓	✓							
M. longicorne A.Gray	6*x*	USA, Arizona, Pina Co, 2006; CR & MB, 18826.			✓	FJ697001, FJ697002	FJ697099	GU216569	GU216346		
M. nayaritense Stuessy	4*x*	M, Nayarit; JV & Spooner, 713							GU216420, GU216422		
M. nayaritense Stuessy	4*x*	M, Nayarit, 2008; JV & EO, 1575.	✓	✓	✓	FJ696992	FJ697091	GU216571			
M. nayaritense Stuessy	4*x*	M, Nayarit, 2008; JV & EO, 1577.	✓	✓	✓	FJ696994, FJ696995, FJ696996	FJ697090	GU216573	GU216411-GU216419, GU216421, GU216423-GU216427	GU216456-GU216458	GU216532-GU216534
M. nayaritense Stuessy	4*x*	M, Nayarit, 2008; JV & EO, 1579.	✓			FJ696993	FJ697092	GU216572			
M. pringlei B.L.Rob.	6*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18634			✓					GU216465	GU216530
M. pringlei B.L.Rob.	6*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18637.		✓	✓	FJ696990, FJ696991	FJ697097	GU216566	GU216389-GU216396	GU216463, GU216464	GU216519-GU216521, GU216525-GU216529
M. pringlei B.L.Rob.	6*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18650.	✓	✓	✓	FJ696988	FJ697094			GU216466-GU216468	GU216522-GU216524, GU216548

Table 1. continued

Taxon	Ploidy level	Collection details, voucher numbers	Genome size	FISH	ITS RE	GenBank accession numbers					
						ITS	*mat*K	*psb*A-*trn*H	5S rDNA	*Pgi*CI	*Pgi*CII
M. pringlei B.L.Rob.	6*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18636			✓						
M. sericeum Lag.	6*x*	M, Michoacán, 2005; TS, JV, CR & IC, 18572.		✓	✓	FJ696986, FJ696987	FJ697093	GU216565	GU216397-GU216401	GU216472-GU216482	GU216511, GU216518, GU216549
M. sericeum Lag.	6*x*	M, Jalisco, 2005; TS, JV, CR, & IC, 18605.			✓						
M. sericeum Lag.	6*x*	M, Oaxaca, 2005; TS, JV, CR, & IC, 18620.	✓	✓							
M. sericeum Lag.	6*x*	M, Michoacán, 2005; TS, JV, CR, & IC, 18625.			✓						
M. sericeum Lag.	6*x*	M, Michoacán, 2005; TS, JV, CR, & IC, 18584.			✓						
M. strigosum Stuessy	4*x*	USA, Texas, Jeff Davis Co, 2005; CR & ML, 18728.		✓	✓	FJ696997, FJ696998	FJ697095	GU216567	GU216381-GU216388	GU216459-GU216462	GU216513, GU216516, GU216517
M. strigosum Stuessy	4*x*	M, Queretaro, 2006; TS, JV & CB, 19073.	✓	✓	✓	FJ696999	FJ697096	GU216568			
Ser. *Glabribracteata*											
M. glabribracteatum Stuessy	2*x*	M, Oaxaca, 2005; TS, JV, CR & IC, 18654.	✓	✓	✓	FJ696989	FJ697100	GU216564	GU216370-GU216380	GU216483	GU216514, GU216515
Ser. *Cupulata*											
M. appendiculatum B.L.Rob.	2*x*	M, Sonora, 2006; TS, JV & CB, 19046.	✓	* ✓	✓	FJ697030	FJ697116	GU216576	GU216355-GU216358	GU216500	GU216504, GU216509
M. cupulatum A.Gray	2*x*	M, Sinaloa, 2006; TS, JV & CB, 19044.		✓	✓	FJ697031	FJ697114	GU216581	GU216318 - GU216324	GU216501	GU216502, GU216503, GU216507, GU216508

Table 1 continued

Taxon	Ploidy level	Collection details, voucher numbers	Genome size	FISH	ITS RE	GenBank accession numbers					
						ITS	*mat*K	*psb*A-*trn*H	5S rDNA	*Pgi*CI	*Pgi*CII
M. cupulatum A.Gray	2*x*	M, Sonora, 2006; TS, JV, & CB, 19045.	✓								
M. cupulatum A.Gray	2*x*	M, Sonora, 2006; TS, JV & CB, 19048.		✓		FJ697032	FJ697115	GU216580			
M. moctezumum B.L.Turner		M, Sonora, 2006; TD & AR, 2007-706 (TEX).				FJ789805, FJ789806	FJ789803				
M. moctezumum B.L.Turner		M, Sonora, 2003; TD & AR, 2003-1228 (TEX).									
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV, CB & EO, 19025.				FJ697026		GU216582	GU216340-GU216343	GU216488-GU216491	GU216510
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV, CB & EO, 19036.			✓	FJ697025	FJ697121				
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV & CB, 19043.				FJ697023, FJ697024	FJ697122				
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV & CB, 19049.				FJ697022		GU216583			
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV & CB, 19050.		✓							
M. rosei B.L.Rob.	2*x*	M, Sinaloa, 2006; TS, JV CR & CB, 19051.	✓								
M. sinuatum Brandegee	2*x*	M, Baja California, 2006; TS & JV, 19037.			✓	FJ697029	FJ697136	GU216579	GU216311-GU216317, GU216431	GU216492-GU216499	
M. tenellum Hook.f. & Arn.	2*x*	M, Nayarit, 2006; TS, JV, CB & EO, 19020.	✓	✓		FJ697028	FJ697117	GU216578			
M. tenellum Hook.f. & Arn.	2*x*	M, Nayarit, 2006; TS, JV, CB & EO, 19022.		✓							
M. tenellum Hook.f. & Arn.	2*x*	M, Nayarit, 2006; TS, JV, CB & EO, 19023.			✓	FJ697027	FJ697118	GU216577	GU216331-GU216339	GU216484-GU216487	GU216506

Table 2. Species names, voucher numbers, and ploidy levels of the analyzed taxa; Accessions marked with an asterix * have also been also used for sequencing. *Glycine max* has been used as internal standard for genome size estimation, unless otherwise indicated ([1]*Pisum sativum*); [2]expected additive values of genome size for polyploids.

Taxon, voucher number	Ploidy level	Genome size		
		1C (pg) ±SD	1Cx (pg)	1C (pg) expected[2]*
Series *Melampodium*				
M. americanum L. 18583	2*x*	1.04 ±0.003[1]	1.04	–
M. americanum L. 19009	2*x*	1.15 ±0.005[1]	1.15	–
**M. diffusum* Cass. 18669	2*x*	1.12 ±0.003[1]	1.12	–
M. diffusum Cass. 18671	2*x*	1.13 ±0.004[1]	1.13	–
M. linearilobum DC. 18662	2*x*	0.49 ±0.008[1]	0.49	–
**M. longipes* (A.Gray) B.L.Rob. 18621	2*x*	1.11 ±0.009[1]	1.11	–
**M. longipes* (A.Gray) B.L.Rob. 19015	2*x*	1.12 ±0.006[1]	1.12	–
**M. mayfieldii* B.L.Turner 19019	4*x*	2.25 ±0.027[1]	1.13	1.94–2.08
**M. pilosum* Stuessy 18587	2*x*	1.05 ±0.006[1]	1.05	–
M. pilosum Stuessy 19010	2*x*	1.03 ±0.002[1]	1.03	–
Series *Sericea*				
M. longicorne A.Gray 18824	6*x*	3.81 ±0.006	1.27	3.76–3.80
**M. nayaritense* Stuessy 1575	4*x*	1.49 ±0.007[1]	0.75	1.53–1.61
**M. nayaritense* Stuessy 1577	4*x*	1.52 ±0.006[1]	0.76	1.53–1.61
**M. nayaritense* Stuessy 1579	4*x*	1.50 ±0.003[1]	0.75	1.53–1.61
**M. pringlei* B.L.Rob. 18650	6*x*	3.28 ±0.040	1.09	3.34
M. sericeum Lag. 18620	6*x*	3.23 ±0.021	1.08	3.34
**M. strigosum* Stuessy 19073	4*x*	2.85 ±0.018	1.43	2.89–2.97
Series *Glabribracteata*				
**M. glabribracteatum* Stuessy 18654	2*x*	1.85 ±0.006	1.85	–
Series *Cupulata* s.str.				
**M. appendiculatum* B.L.Rob. 19046	2*x*	0.95 ±0.002	0.95	–
M. cupulatum A.Gray 19045	2*x*	0.93 ±0.001	0.93	–
M. rosei B.L.Rob. 19051	2*x*	0.91 ±0.001	0.91	–
**M. tenellum* Hook.f. & Arn. 19020	2*x*	0.92 ±0.009	0.92	–

initial denaturation at 95 °C for 4 min, followed by 35 cycles of 1 min at 95 °C, 30 sec at 55 °C, 45 sec at 72 °C, and final elongation step at 72 °C for 10 min.

The region between exons 11 and 16 of the low copy nuclear gene *Pgi*C were amplified using degenerate primers AA11F and AA16R (Ford *et al.*, 2006) with the following PCR conditions: 94 °C for 3 min, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 54 °C, 2 min at 72 °C, and final elongation at 72 °C for 10 min. Analyses of these products allowed the development of the *Melampodium*-specific primers 11f-Melampodium (5'-GGAGGYMGATAYRGYGGTAAG-3') and 16R-Melampodium (5'-CRTTRCTYTCCATGCTMACCTAHA-3') used for further amplifications. PCR was carried out with the same reagents mix as for the 5S rDNA spacer region, but with 1μM of each primer to compensate for their degeneration. PCR conditions were as follows: 90 °C for 5 min, followed by 35 cycles of 30 sec at 94 °C, 1 min at 54 °C, 3 min at 69 °C, and final elongation at 69 °C for 20 min and at 72 °C for 10 min.

The PCR products of the 5S rDNA spacer and the partial *Pgi*C were cloned into pGEM-T Easy vector system and transformed into JM109 competent cells (Promega, Madison, WI, USA) according to the manufacturer's instructions. Inserts of 6–18 positive clones for the 5S rDNA spacer region (depending on the ploidy level with six clones per diploid genome on average) and of 10–20 clones per diploid genome for *Pgi*C (due to the presence of two paralogues) were amplified using colony PCR with universal M13 primers, whereby recombinant colonies were added directly into the PCR reaction and inserts amplified using reagents and conditions described in Park *et al.* (2007). Colony PCR products were purified using *E. coli* Exonuclease I and Calf Intestine Alkaline Phosphate (CIAP; MBI-Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The purified DNA fragments were directly sequenced using dye terminator chemistry following the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The cycle sequencing reactions were

performed using M13 universal primers, either in one or in both directions. Sequencing reactions were run on a 3130xl Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were assembled in AutoAssembler 1.4.0 (Applied Biosystems, Foster City, CA, USA).

Alignment and phylogenetic analysis –Alignments were generated with Muscle 3.6 (Edgar, 2004) using default settings and improved by visual refinement using the program BioEdit 7.0.9.0 (Hall, 1999). All sequences are deposited in GenBank (Table 1). Regions of ambiguous alignment were excluded from the analyses. Due to high sequence variation of 5S rDNA spacer sequences resulting in some regions with rather ambiguous alignment, three datasets were originally tested for maximum parsimony: (1) dataset aligned with G-Blocks v.0.91b (Castresana, 2000) setting the parameters to minimum numbers of sequences for conserved positions to 63, for flanking positions to 106, the maximum number of contiguous non-conserved positions to 8, minimum length of a block to 10 and allowed gap position to half, where 409 characters of the originally 889 aligned positions were retained for analysis, (2) a dataset with manually excluded ambiguously aligned positions retaining 713 aligned characters and (3) a dataset with 377 characters where only clearly unambiguously aligned positions (according to visual inspection) were retained. The analyses of the three datasets yielded very similar results varying only slightly in resolution and support values. Thus, the first dataset (G-Blocks assisted alignment) was chosen for further analyses, as this approach has been the most objective. The plastid datasets (*mat*K and *psb*A-*trn*H) were combined for analyses. A large inversion (alignment positions 60-113) within *psb*A-*trn*H region was re-inverted for the analyses.

Two paralogues of the *Pgi*C gene, named from now on *Pgi*CI and *Pgi*CII have been recovered from all species (Neighbor Joining analysis). Nucleotide positions of the intron between exons 15 and 16 were deleted from both analysed *Pgi*C datasets (*Pgi*CI:

characters 714-749; *Pgi*CII: characters 692-744), due to poly-Ts and other microsatellites presence which rendered the alignment unreliable. ITS was analyzed with indels treated as missing data. Maximum parsimony analyses were performed using PAUP* 4.0b10 (Swofford, 2003) treating all characters as equally weighted. Heuristic searches included 1,000 replicates of random sequence addition, tree bisection reconnection (TBR) branch swapping, and MulTrees on, but permitting no more than 10 trees to be held in each step. Nodal support was assessed via bootstrap values (BS; Felsenstein, 1985), which were calculated using PAUP* 4.0b10 with 10,000 bootstrap replicates each with 20 random sequence addition replicates holding maximally 10 trees per replicate, SPR branch swapping, and MulTrees on.

The Bayesian analyses were conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best-fit substitution models were determined using MrModeltest 2.2 (Nylander, 2005, program distributed by the author, Uppsala University, Uppsala). For the details of the ITS analyses refer to Blöch *et al.* (2009). The two paralogues of the low copy nuclear gene *Pgi*C were analyzed separately using partitions separating exon and intron positions. For *Pgi*CI (paralogue I) HKY substitution model was selected for the exons and HKY+Γ for the introns. For *Pgi*CII (paralogue II) exons were analyzed using the HKY+Γ model and introns were analyzed employing GTR+Γ model (HKY+Γ model has also been tested, but both analyses yielded very similar results varying only slightly in posterior support values). The 5S rDNA intergenic spacer dataset was analyzed with the HKY+Γ substitution model. The combined plastid dataset included *trn*K intron partition, the combined first and second codon position of the *mat*K gene (both partitions analysed with a F81 + Γ model), the third codon position of the *mat*K (analysed with a GTR + Γ model), and the *psb*A-*trn*H partition analysed with the HKY+Γ substitution model. The MCMC settings for all Bayesian analyses consisted of four runs with four chains each (three heated ones using

the default heating scheme) for 5×10^6 generations sampling every 1,000th generation, using default priors and estimating all parameters during the analysis. The first 10% of each run, which was after the chains had reached stationarity as judged from plots of the likelihood and of all parameters and from split variances being <0.01, were discarded as burn-in. A majority rule consensus tree was constructed from the posterior set of 18,000 trees. Trees were rooted using species of ser. *Cupulata* s.str.

To depict reticulate relationships among species, we used *Pgi*CI multilabelled-tree representation in the PADRE software (Huber *et al.*, 2006; Lott *et al.*, 2009). Input tree for PADRE was the 95 percent majority rule consensus tree retained from the Bayesian analysis.

Chromosome analyses and Fluorescence *in situ* hybridization (FISH) – Chromosome numbers and karyotypes of all *Melampodium* accessions used in this study have been determined using standard Feulgen staining (Weiss-Schneeweiss *et al.*, 2009). FISH was applied to all but two species (diploid *M. moctezumum* and *M. sinuatum*, ser. *Cupulata*; due to lack of viable seeds). Chromosomes (5-10 seedlings of each accession; Table 1) were prepared by enzymatic digestion as described in Weiss-Schneeweiss *et al.* (2008). Fluorescence *in situ* hybridization (FISH) and detection were carried out according to the methods of Schwarzacher & Heslop-Harrison (2000) and Weiss-Schneeweiss *et al.* (2008) with minor modifications. Probes used for FISH were 18S rDNA from *Arabidopsis thaliana* in plasmid pSK+, and 5S rDNA genic region isolated from *Melampodium montanum* in plasmid pGEM-T Easy, labelled with biotin or digoxygenin (Roche, Vienna, Austria), respectively. Probes were labelled either directly by PCR (5S rDNA) or using a nick translation kit (18S rDNA; Roche, Vienna, Austria) and detected with either Extravidin-Cy3 (for biotin; Sigma, Vienna, Austria) or anti-digoxygenin-FITC (for digoxygenin; Roche, Vienna, Austria). Analyses of preparations were performed with an Axioplan2 epifluorescent microscope (Carl Zeiss,

Vienna, Austria), images acquired with a CCD camera and files processed using Axiovision ver. 3.5 (Carl Zeiss, Vienna, Austria). For rDNA localization, a minimum of 30 well-spread metaphases and prometaphases were analysed for each species. Contrast of the images was adjusted using only those fuctions that apply to the whole image equally. Chromosomes carrying rDNA were cut out of the images and contrasted to clearly visualize the loci.

Genome size - Genome size was analyzed for 23 populations of 17 species of sect. *Melampodium* (Table 2). Approximately 10 mm^2 leaf tissue of *Glycine max* 'Idefix' (GM; 1C = 1.28 pg, Doležel *et al.*, 1998) or *Pisum sativum* (PS; 1C = 4.42 pg, Greilhuber & Ebert, 1994) and two seedlings of *Melampodium* were chopped together in 500 µL cold iso-buffer. After adding another 500 µL iso-buffer the nuclei solution was filtered trough a 30 µL nylon mesh. 50 µL of RNase A (3 mg/mL; MBI-Fermentas, St. Leon-Rot, Germany) were added to each sample and incubated at 37 °C for 30 min to remove RNA. The samples were incubated for c. 90 min in propidium iodide (PI) solution (0.1 mM; Sigma, Vienna, Austria). Analysis was conducted with a Partec CyFlow ML (Partec GmbH, Münster, Germany; equipped with green laser beam) and each sample was re-measured four times to check for value accuracy. Due to lack of material of *M. moctezumum* genome size values were estimated via flow cytometry of non-germinating seeds and values corrected for DNA condensation degree difference using *M. pilosum* and *M. sinuatum* seeds as reference (J. Rauchova & H. Weiss-Schneeweiss, unpublished).

Amplification and restriction digest of ITS1 and ITS2 – Restriction analysis of ITS1 and ITS2 regions was performed to obtain information on genomic representation of all types of ITS sequences within the analysed genomes. ITS sequences available from a previous study (Blöch *et al.,* 2009) were used for screening for appropriate restriction enzymes using SMS (Stothard, 2000;

www.ualberta.ca/~stothard/javascript/rest_map.html). ITS1 and ITS2 regions were amplified from 17 species (1-3 accessions per species) using primers anchored in 18S and 5.8S rDNA and 5.8S and 26S rDNA regions, respectively (Blöch *et al.*, 2009). The ITS1 and 2 amplification followed the protocol of Blöch *et al.* (2009). The ITS1 region was digested with FastDigest® *Taq*I (MBI-Fermentas, St. Leon-Rot, Germany) at 65 °C for 5 min according to the manufacturer's protocol. The ITS2 region was digested with (1) the FastDigest® *Hae*III restriction enzyme (MBI-Fermentas, St. Leon-Rot, Germany) at 37 °C for 10 min, and (2) with FastDigest® *Bfa*I (MBI-Fermentas, St. Leon-Rot, Germany) for 10 min at 37 °C, followed by a thermal inactivation at 80 °C for 5 min, according to the manufacturer's recommendation. Resulting DNA fragments were separated electrophoretically (60 min at 80V) on a 2% agarose gel.

Results

Species relationships

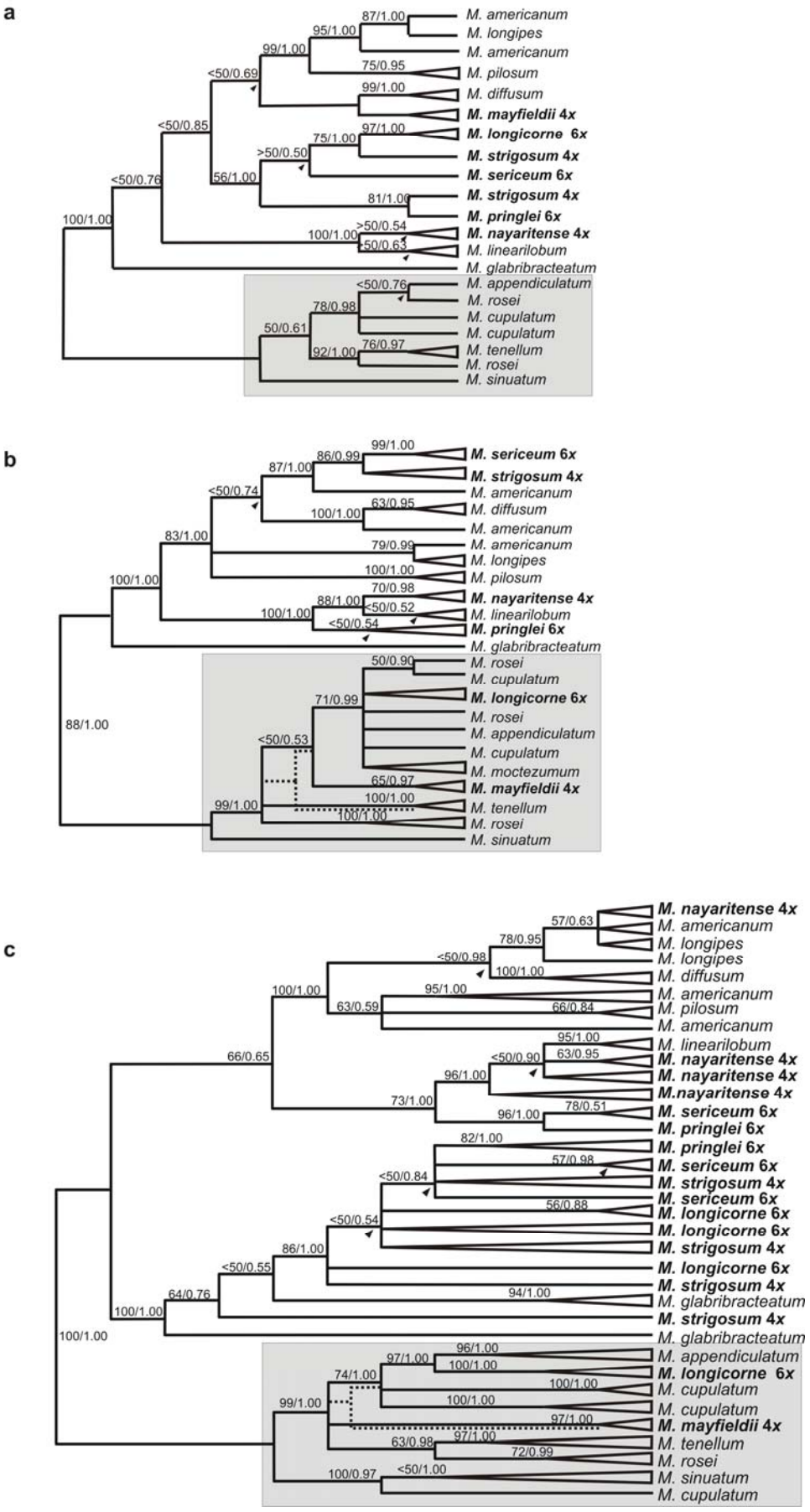
Combined analyses of plastid regions (*psb*A-*trn*H and *mat*K) - The results of the analyses of *mat*K and the *psb*A-*trn*H datasets were largely congruent; thus, the final analysis has been performed on a combined dataset including 2,431 nucleotide characters (473 bps of *psb*A-*trn*H and 1,958 bps of *mat*K), 124 of which were variable and 94 were parsimony-informative. Twenty-nine accessions from 17 species of sect. *Melampodium* (sers. *Cupulata* s.str., *Glabribracteata*, *Melampodium*, *Sericea*) were analyzed resulting in 153 trees with a score of 164 (CI excluding parsimonious uninformative characters 0.73 and RI 0.93). The strict consensus tree was topologically very similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –4,468.71; Fig. 1a).

In the plastid-derived phylogeny tetraploid *M. mayfieldii* was placed within ser. *Melampodium* (excluding *M. linearilobum*; BS/PP <50/0.69), albeit with low support.

Four polyploids of ser. *Sericea*, hexaploid *M. longicorne*, *M. pringlei*, *M. sericeum* and the tetraploid *M. strigosum*, formed a monophyletic group (BS/PP 56/1.00), whereas the fifth polyploid of the series, *M. nayaritense*, was recovered as sister to the diploid *M. linearilobum* (BS/PP 100/1.00). The diploids of ser. *Cupulata* s.str. formed a monophyletic group (BS/PP 100/1.00). *Melampodium glabribracteatum* (ser. *Glabribracteata)* was recovered as the sister taxon (albeit unsupported) to sers. *Melampodium* and *Sericea*.

nrITS – The results of the nuclear ITS dataset analyses (for details see Blöch & al., 2009; Fig. 1b) have only partly been congruent with the clades recovered from the plastid phylogeny. Hexaploid *M. longicorne* (ser. *Sericea*) and tetraploid *M. mayfieldii* (ser. *Melampodium*) were nested within a well supported clade of diploids of ser. *Cupulata* s.str. (BS/PP 88/1.00). *Melampodium longicorne* grouped in a polytomy with sequences of *M. rosei*, *M. appendiculatum*, *M. cupulatum* and *M. moctezumum* (BS/PP 71/0.99). *Melampodium mayfieldii* did not tie to any particular taxon within ser. *Cupulata.* The diploid *M. linearilobum* (ser. *Melampodium*) grouped with the tetraploid *M. nayaritense* (BS/PP 88/1.00), and with hexaploid *M. pringlei* (BS/PP 100/1.00; both ser. *Sericea*). *Melampodium sericeum* (6*x*) and *M. strigosum* (4*x*; both ser. *Sericea*) were nested within diploid species of ser. *Melampodium* (BS/PP 83/1.00), tying to *M. americanum* (BS/PP 87/1.00).

5S rDNA NTS - The analysis of the nuclear NTS of the ribosomal 5S rRNA gene included 409 nucleotide characters of the original 883 characters after reduction of the matrix with G-Blocks v.0.91b (Castresana, 2000). 292 of 353 variable characters were parsimony-informative. 125 clones representing 18 accessions and 17 species of sect. *Melampodium* were included in the analysis, which resulted in 6,730 trees with a


a
87/1.00
95/1.00
99/1.00
75/0.95
99/1.00
97/1.00
75/1.00
81/1.00
>50/0.54
100/1.00
<50/0.63
<50/0.76
78/0.98
50/0.61
92/1.00
76/0.97
<50/0.69
<50/0.85
>50/0.50
56/1.00
<50/0.76
100/1.00
M. americanum
M. longipes
M. americanum
M. pilosum
M. diffusum
M. mayfieldii 4x
M. longicorne 6x
M. strigosum 4x
M. sericeum 6x
M. strigosum 4x
M. pringlei 6x
M. nayaritense 4x
M. linearilobum
M. glabribracteatum
M. appendiculatum
M. rosei
M. cupulatum
M. cupulatum
M. tenellum
M. rosei
M. sinuatum
b
99/1.00
86/0.99
87/1.00
<50/0.74
63/0.95
100/1.00
83/1.00
79/0.99
100/1.00
100/1.00
70/0.98
88/1.00
100/1.00
<50/0.52
<50/0.54
88/1.00
50/0.90
71/0.99
<50/0.53
65/0.97
100/1.00
99/1.00
100/1.00
M. sericeum 6x
M. strigosum 4x
M. americanum
M. diffusum
M. americanum
M. americanum
M. longipes
M. pilosum
M. nayaritense 4x
M. linearilobum
M. pringlei 6x
M. glabribracteatum
M. rosei
M. cupulatum
M. longicorne 6x
M. rosei
M. appendiculatum
M. cupulatum
M. moctezumum
M. mayfieldii 4x
M. tenellum
M. rosei
M. sinuatum
c
57/0.63
78/0.95
<50/0.98
100/1.00
100/1.00
95/1.00
63/0.59
66/0.84
66/0.65
95/1.00
63/0.95
<50/0.90
96/1.00
73/1.00
78/0.51
96/1.00
82/1.00
57/0.98
<50/0.84
56/0.88
<50/0.54
86/1.00
<50/0.55
64/0.76
94/1.00
100/1.00
100/1.00
96/1.00
97/1.00
100/1.00
74/1.00
100/1.00
99/1.00
100/1.00
97/1.00
63/0.98
97/1.00
72/0.99
100/0.97
<50/1.00
M. nayaritense 4x
M. americanum
M. longipes
M. longipes
M. diffusum
M. americanum
M. pilosum
M. americanum
M. linearilobum
M. nayaritense 4x
M. nayaritense 4x
M.nayaritense 4x
M. sericeum 6x
M. pringlei 6x
M. pringlei 6x
M. sericeum 6x
M. strigosum 4x
M. sericeum 6x
M. longicorne 6x
M. longicorne 6x
M. strigosum 4x
M. longicorne 6x
M. strigosum 4x
M. glabribracteatum
M. strigosum 4x
M. glabribracteatum
M. appendiculatum
M. longicorne 6x
M. cupulatum
M. cupulatum
M. mayfieldii 4x
M. tenellum
M. rosei
M. sinuatum
M. cupulatum

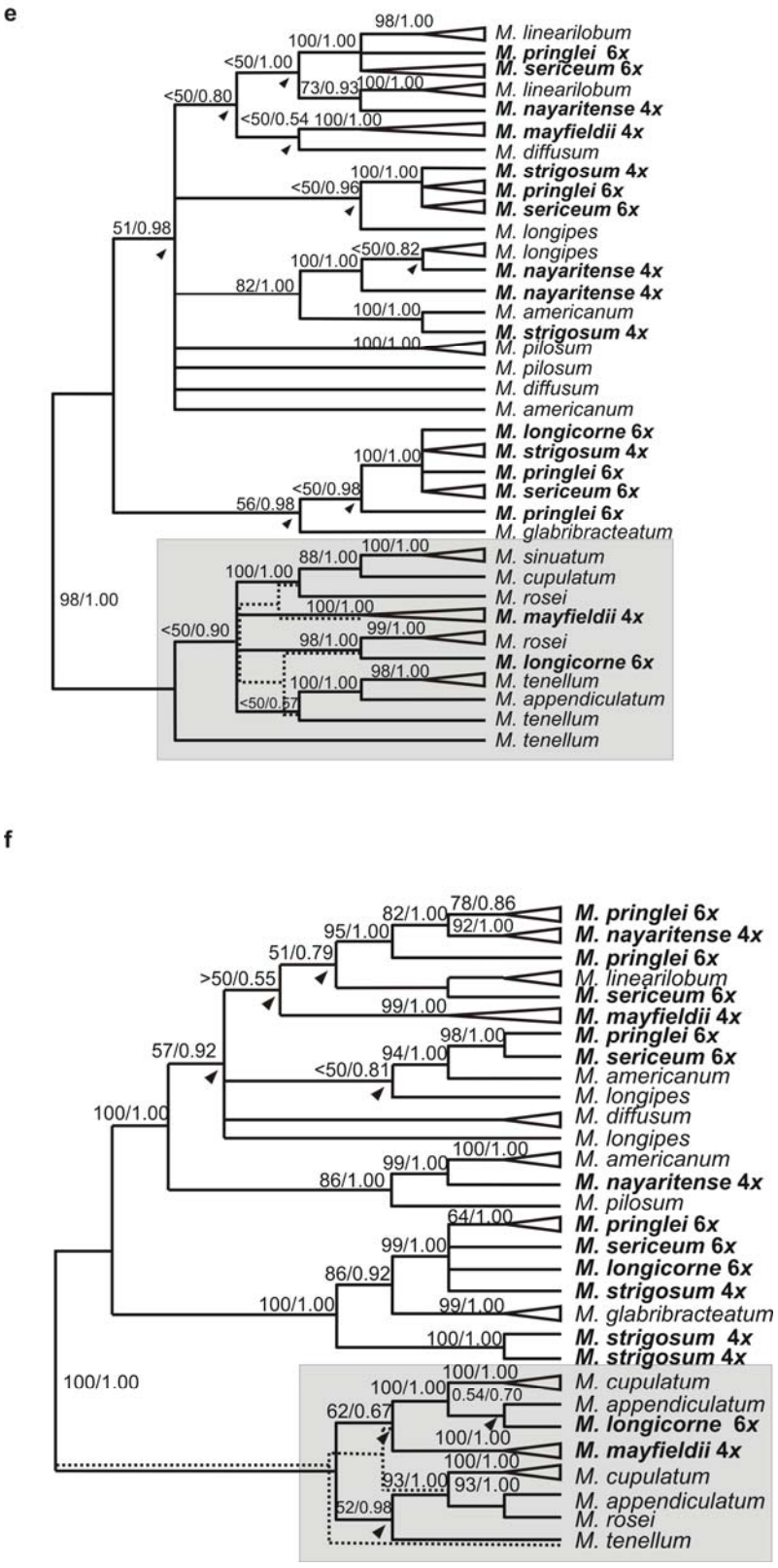


Fig. 1. Phylogenetic relationships of nuclear and plastid DNA markers of the species of *Melampodium* sect. *Melampodium* sers. *Melampodium*, *Sericea*, *Cupulata* s.str. and *Glabribracteata* inferred from Bayesian (solid lines) and maximum parsimony analysis (dotted lines). **a,** combined plastid *mat*K and *psb*A-*trn*H spacer; **b**, nuclear ITS; **c**, nuclear 5S rDNA NTS; **d**, nuclear low copy gene *Pgi*CI; **e**, nuclear low copy gene *Pgi*CII. Branches collapsing in the strict consensus tree of the maximum parsimony analysis are indicated by arrowheads. Numbers at nodes are bootstrap values/posterior probabilities. Ploidy level is indicated for polyploid taxa (bold). The grey box indicates diploids of ser. *Cupulata*.

score of 966 (CI excluding parsimonious uninformative characters 0.60 and RI 0.95). The strict consensus tree was topologically very similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –4,315.38; Fig. 1c). The 5S rDNA NTS phylogeny provided more detailed insight into the relationships of the allopolyploids, as rDNA repeat types of loci of different parental origin were identified in nearly all allopolyploids. Two types of 5S rDNA NTS sequences were found in tetraploid *M. nayaritense*, one grouping with the diploid *M. americanum* and *M. longipes* (BS/PP 78/0.95), and the other with the diploid *M. linearilobum* (BS/PP 96/1.00). The latter group included also one of the copies of 5S rDNA NTS of hexaploid *M. sericeum* and *M. pringlei* (BS/PP 73/1.00). The second type of 5S rDNA NTS present in the two latter hexaploids grouped with *M. strigosum* (4*x*, for which only one parental copy type was found), *M. longicorne* (6*x*), and with the diploid *M. glabribracteatum* (BS/PP 100/1.00). The second type of 5S rDNA NTS recovered from hexaploid *M. longicorne* clustered within ser. *Cupulata* being sister to *M. appendiculatum* (BS/PP 97/1.00). Only one type of 5S rDNA NTS sequence was found in the tetraploid *M. mayfieldii* and grouped with diploids of ser. *Cupulata* s.str. (BS/PP 99/1.00), not tying to any of its taxa in particular.

Low copy nuclear *Pgi*C - *Pgi*CI - The matrix of the paralogue I of the low copy nuclear gene *Pgi*C (*Pgi*CI) included 68 clones of 19 accessions and 17 species. Thirty-six of 775 characters were excluded from the analyses (position 714-749) due to alignment ambiguities caused by microsatellite presence. The dataset included 365 variable characters of which 236 were parsimonious informative. Maximum parsimony analysis resulted in 3,983 trees with a score of 649 (CI excluding parsimony-uninformative characters 0.63 and RI 0.89). The strict consensus tree is topologically very similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –4,923.23; Fig. 1d). For most of the allopolyploids a number of

homeologous sequences corresponding to the ploidy level were detected (two homologues in tetraploid, three in hexaploids). One homeologue *Pgi*CI of *M. nayaritense* (4*x*) grouped with the diploid *M. linearilobum* (BS/PP 73/0.93) and the second one with the diploid *M. longipes* (BS/PP 100/1.00). The two homeologues of *M. strigosum* (4*x*) grouped either with diploid *M. longipes*/*M. americanum* or with *M. glabribracteatum* (BS/PP56/0.98). The first of the aforementioned homeologues of *M. strigosum* (4*x*) exhibited some variation on the level of sequences, and two subtypes of this homeologue were recovered, one grouping with diploid *M. longipes* (BS/PP <50/0.96), and the other with closely related diploid *M. americanum* (BS/PP 100/1.00). The first two homeologues of *M. pringlei* (6*x*) and *M. sericeum* (6*x*) grouped with two homeologues of *M. strigosum* (4*x;* BS/PP 100/1.00; BS/PP <50/0.98) respectively. The remaining third homeologue of *M. pringlei* (6*x*) and *M. sericeum* (6*x*) grouped with diploid *M. linearilobum* (BS/PP 100/1.00). The two homeologues of *M. longicorne* (6*x*) grouped either with diploid species of ser. *Cupulata* s.str., sister to *M. rosei* (BS/PP 98/1.00), or with one of the *M. strigosum* (4*x*) homeologues (BS/PP 100/1.00). The third homeologue of *M. longicorne* (expected to originate from the *M. strigosum* genome) has not been recovered (likely due to the limited number of analysed clones). Two homeologues have been recovered for tetraploid *M. mayfieldii*, one grouping with diploid *M. diffusum* (albeit with very low support BS/PP <50/0.54) and the other in unresolved, isolated position within diploid taxa of ser. *Cupulata* s.str.

*Pgi*CII – The dataset of the second *Pgi*C paralogue (*Pgi*CII) included 52 clones of 18 accessions of 17 species. One hundred-thirty of 768 aligned character positions were excluded using G-Blocks v.0.91b (Castresana, 2000). The remaining dataset included 288 variable characters of which 188 were parsimony-informative. Maximum parsimony analyses resulted in 88 trees with a score of 424 (CI excluding parsimonious uninformative characters 0.71 and RI 0.93). The strict consensus tree was topologically

very similar to the majority rule consensus tree from the Bayesian analysis (harmonic mean –ln = –3,895.54; Fig. 1e). The *Pgi*CII phylogeny was congruent with the *Pgi*CI phylogeny. Most polyploids contained homeologous sequences, which number corresponded with ploidy level. One of the homeologues of the tetraploid *M. nayaritense* grouped with the hexaploid *M. pringlei* (BS/PP 82/1.00), and with a clade composed of diploid *M. linearilobum* and the hexaploid *M. sericeum*, albeit with low support (51/0.79). The second homeologue of *M. nayaritense* (4*x*) was recovered as sister to the diploids *M. americanum* and *M. pilosum* (BS/PP 86/1.00). The second homeologue of each hexaploid *M. pringlei* and *M. sericeum* was sister to *M. americanum* (2*x*; BS/PP 94/1.00), and with low support to *M. longipes* (2*x*; BS/PP >50/0.81), whereas their third homeologues grouped with *M. strigosum* (4*x*), *M. longicorne* (6*x*) and the diploid *M. glabribracteatum* (BS/PP 100/1.00). The second homeologue of *M. strigosum* (4*x*) was not recovered within the sequenced clones. Similarly, only two out of the expected three homeologues have been recovered from the hexaploid *M. longicorne*, one grouping with the other polyploids of ser. *Sericea* and the diploid *M. glabribracteatum* and the second one being sister to diploid *M. appendiculatum* or *M. cupulatum* of ser. *Cupulata* (BS/PP 100/1.00). Finally, the two homeologues of tetraploid *M. mayfieldii* grouped either at an unresolved position within the diploids of ser. *Melampodium* and the polyploids of ser. *Sericea* (BS/PP 100/1.00) or at also an unresolved position within diploids of ser. *Cupulata* s.str. (BS/PP 100/1.00). Some variation between *Pgi*CII copies of *M. americanum* and *M. longipes*, as well as most of the taxa of series *Cupulata* s.str. suggests either their frequent hybridization or local duplication of this paralogue.

Origin of polyploids – Analyses of reticulate relationships and the origin of putative allopolyploids were performed with PADRE using the *Pgi*CI dataset. All polyploids were recovered as allopolyploids (Fig. 2). Comparisons of species

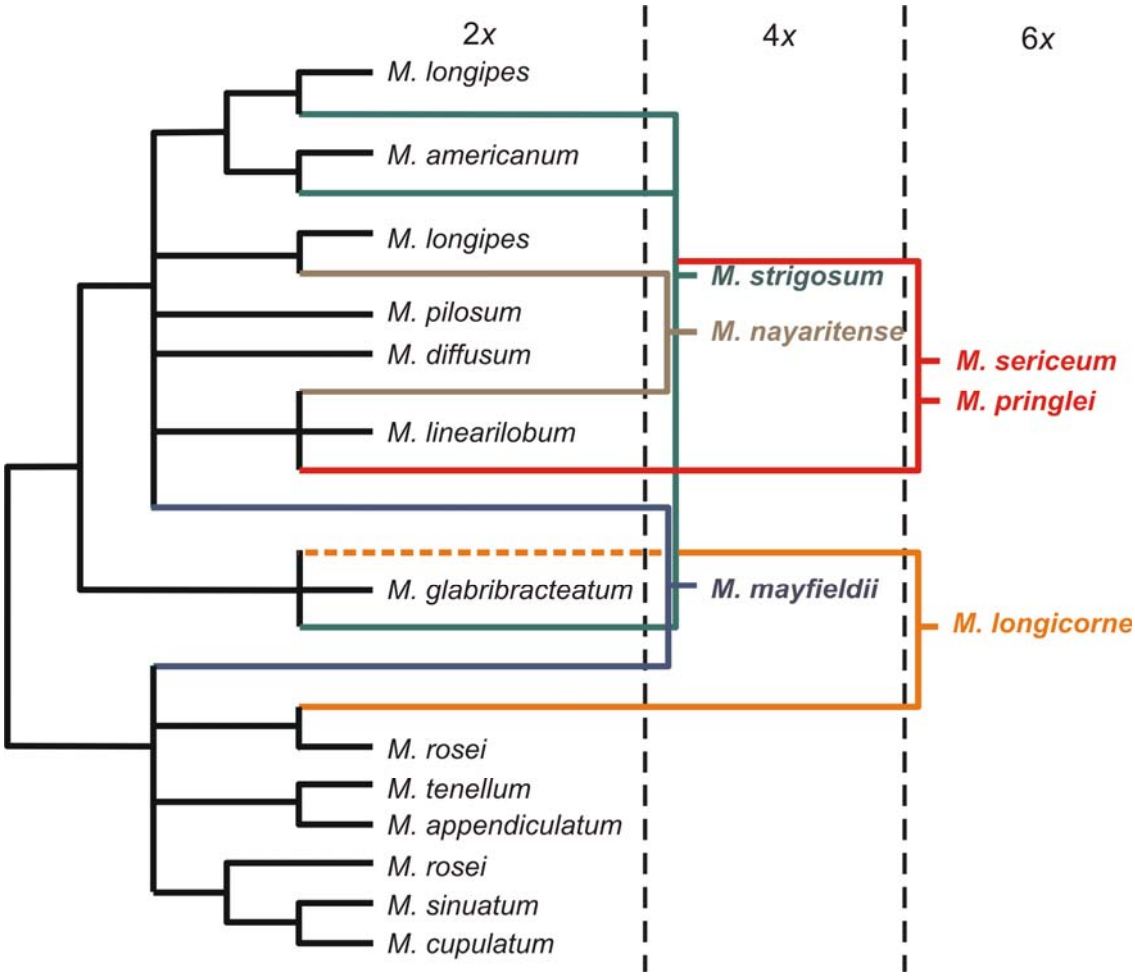


Fig. 2. Inference of the parental taxa of the allopolyploid species of sect. *Melampodium* based on low copy *Pgi*CI sequence data using PADRE (Lott *et al*., 2009). In the case of *M. longicorne* the dashed line indicates its origin inferred from *Pgi*CI, where likely one homeologe was missed in the cloning procedure. Therefore the solid line shows its origin from *M. strigosum* as inferred from *Pgi*CII, the 5S rDNA spacer and plastid data.

relationships based on analyses of plastid and nuclear markers allowed the inference of putative maternal and paternal species/species groups (Figs. 1a-e). Tetraploid *M. strigosum* has been inferred to originate from hybridization between *M. glabribracteatum* (or its closely related extinct relative) and *M. americanum*/*M. longipes*. Tetraploid *M. nayaritense* has been confirmed to be of hybrid origin involving *M. linearilobum* (maternal donor of 10 small chromosome pairs) and *M. longipes*/*M. americanum* (paternal parent(s)). Tetraploid *M. mayfieldii* has been demonstrated to be of interserial hybrid origin between as yet unidentified species of ser. *Cupulata* s.str.

(paternal parent) and ser. *Melampodium* (maternal parent). All three remaining hexaploids shared one parental species, tetraploid *M. strigosum* (always acting as maternal parent). *Melampodium pringlei* and *M. sericeum* originated from the same type of cross between tetraploid *M. strigosum* and diploid *M. linearilobum* (paternal parent). The origin of *M. longicorne* involved a cross between *M. strigosum* and one of the diploid species of series *Cupulata* s.str. (paternal parent).

Restriction analysis – Restriction analyses of the ITS1 region (Fig. 3) revealed three groups of taxa: (1) diploid *M. pilosum*, *M. longipes*, and *M. linearilobum* (all ser. *Melampodium*), as well as the tetraploid *M. nayaritense* and the hexaploid *M. pringlei* (ser. *Sericea*), (2) diploid members of ser. *Cupulata* s.str. and ser. *Glabribracteata*, as well as tetraploid *M. mayfieldii* (ser. *Melampodium*) and hexaploid *M. longicorne* (ser. *Sericea*), and (3) *M. americanum* and *M. diffusum* (ser. *Melampodium*) and tetraploid *M. strigosum* and hexaploid *M. sericeum* (ser. *Sericea*). Restriction of the ITS2 region with two enzymes revealed four different patterns shared by: (1) most species of ser. *Melampodium* (except for *M. linearilobum* and *M. mayfieldii*), tetraploid *M. strigosum*, and hexaploid *M. sericeum* (ser. *Sericea*); (2) all species of ser. *Cupulata* s.str., tetraploid *M. mayfieldii* (ser. *Melampodium*), and hexaploid *M. longicorne* (ser. *Sericea*); (3) the diploid *M. linearilobum* (ser. *Melampodium*), tetraploid *M. nayaritense*, and hexaploid *M. pringlei* (ser. *Sericea*); and (4) *M. glabribracteatum* (ser. *Glabribracteata*). Combining the results of both ITS regions revealed five types of restriction site polymorphisms: (1) pattern shared by *M. linearilobum* (2*x*), *M. nayaritense* (4*x*), and *M. pringlei* (6*x*); (2) pattern unique to *M. glabribracteatum* (2*x*); (3) pattern shared by the diploids *M. pilosum* and *M. longipes*; (4) pattern typical for all the species of ser. *Cupulata* s.str., as well as *M. mayfieldii* (4*x*) and *M. longicorne* (6*x*); and (5) pattern of diploid *M. americanum* and *M. diffusum*, as well as *M. strigosum* (4*x*) and *M. sericeum* (6*x*). These data are in agreement with phylogenetic relationships

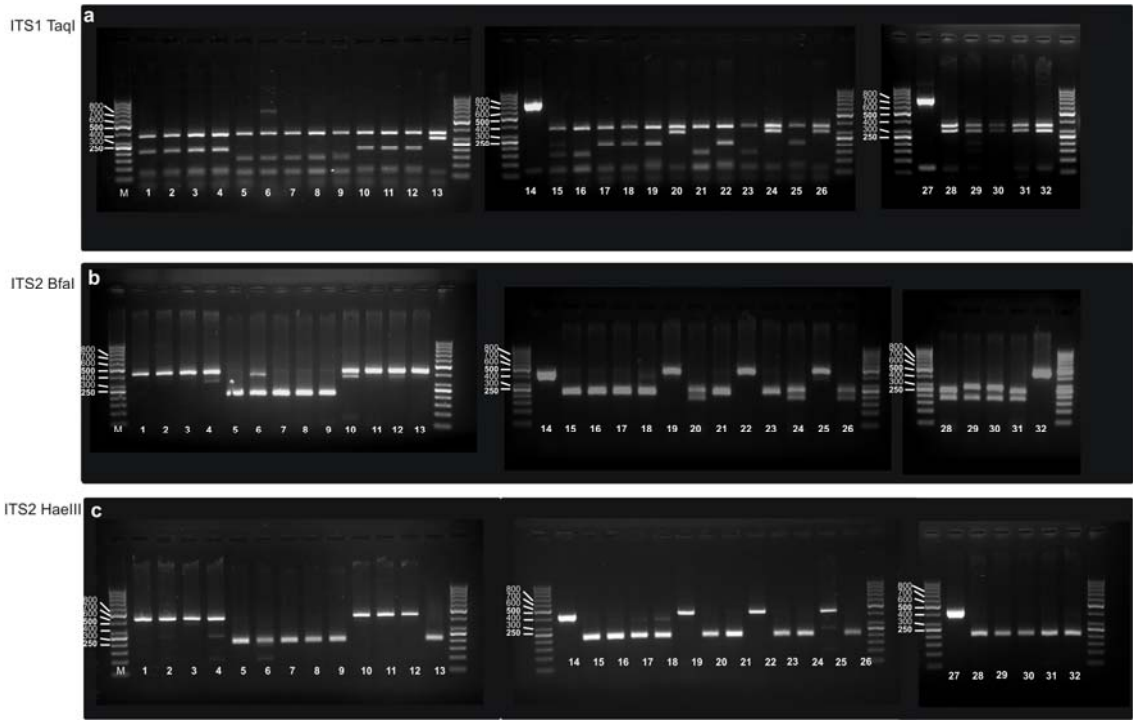


Fig. 3. Restriction pattern of nuclear ITS1 and ITS2 regions in species of sect. *Melampodium* sers. *Cupulata* s.str., *Glabribracteata*, *Melampodium*,and *Sericea*: **a,** ITS1 digestion with *Taq*I; **b**, ITS2 digestion with *Bfa*I; **c**, ITS2 digestion with *Hae*III: Lanes 1–32: M, marker, Gene Ruler^{TM} 50 bp DNA Ladder (MBI-Fermentas, St.Leon-Rot, Germany); 1, *M. linearilobum* 18667, 2*x;* 2, *M. linearilobum* 18661, 2*x;* 3, *M. nayaritense* 1577, 4*x;* 4, *M. nayaritense* 1575, 4*x;* 5, *M. strigosum* 18728, 4*x;* 6, *M. strigosum* 19073, 4*x;* 7, *M. sericeum* 18605, 6*x;* 8, *M. sericeum* 18625, 6*x;* 9, *M. sericeum* 18572, 6*x;* 10, *M. pringlei* 18650, 6*x;* 11, *M. pringlei* 18636, 6*x;* 12, *M. pringlei* 18637, 6*x;* 13, *M. glabribracteatum* 18654, 2*x;* 14, undigested amplification product; 15, *M. americanum* 18609, 2*x;* 16, *M. diffusum* 18666, 2*x;* 17, *M. pilosum* 18587, 2*x;* 18, *M. longipes* 18619, 2*x;* 19, *M. linearilobum* 18662, 2*x;* 20, *M. mayfieldii* 19019, 4*x;* 21, *M. sericeum* 18584, 6*x;* 22, *M. pringlei* 18634, 6*x;* 23, *M. strigosum* 18728, 4*x;* 24, *M. longicorne* 18826, 6*x;* 25, *M. nayaritense* 1575, 4*x;* 26, *M. cupulatum* 19044, 2*x;* 27, undigested amplification product; 28, *M. appendiculatum* 19046, 2*x;* 29, *M. sinuatum* 19037, 2*x;* 30, *M. rosei* 19036, 2*x;* 31, *M. tenellum* 19023, 2*x;* 32, *M. glabribracteatum* 18654, 2*x*.

recovered from sequencing of the ITS region and confirm that most, if not all, types of ITS repeats were recovered from all analysed genomes by cloning and sequencing of the ITS regions.

Chromosomal analyses and rDNA loci localization – Chromosome numbers and karyotypes of all analysed species have recently been published (Weiss-Schneeweiss *et al.*, 2009). Karyotypes of most of the diploid species are symmetrical, and chromosomes are middle-sized. Only two diploid species possess significantly different karyotypes: *M. linearilobum* (ser. *Melampodium*; Fig. 4c) with 2*n* = 20 very small chromosomes and *M. glabribracteatum* (ser. *Glabribracteata*; Fig. 4h) with 2*n* =

20 significantly larger chromosomes. Karyotypes of the tetraploid species ($2n = 4x = 40$) are either fairly symmetrical and unimodal (*M. strigosum* and *M. mayfieldii*) or bimodal with 20 small and 20 bigger chromosomes (*M. nayaritense*; Fig 5i-k). The same pattern is observed among the hexaploid species as *M. pringlei* and *M. sericeum* have strongly bimodal karyotypes ($2n = 6x = 60$) with 40 big and 20 small chromosomes, and *M. longicorne* possesses a fairly symmetrical and unimodal karyotype (Fig. 4l-n).

Localization of 5S and 35S rDNA loci by FISH revealed three distinct patterns among the diploids analysed: (1) two pairs of subterminally localized 35S rDNA loci and one pair of interstitial 5S rDNA loci, all on different chromosomes (*M. glabribracteatum*, ser. *Glabribracteata*, and all diploid species of ser. *Melampodium* except for *M. linearilobum*; Fig. 4a,b,d,h); (2) two subterminal loci of 35S rDNA and one interstitial locus of 5S rDNA, the latter localized on one of the chromosomes carrying the 35S rDNA locus, albeit within its other arm in diploids of ser. *Cupulata* s.str. (Fig. 4e-g); and (3) two subterminal 35S rDNA loci and one subterminal locus of 5S rDNA in *M. linearilobum* (Fig. 4c). The locus of 5S rDNA is on the chromosomes carrying one of the 35S rDNA loci, although on its other chromosomal arm.

Tetraploid *M. strigosum* (Fig. 4k) has two pairs of subterminal 35S rDNA loci and one pair of interstitial 5S rDNA loci, all of which are localized on different chromosomes. This species has thus lost two parental loci of 35S rDNA (at least one of paternal *M. americanum* origin) and one (maternal *M. glabribracteatum*) 5S rDNA locus. Tetraploid *M. nayaritense* (Fig. 4j) possesses two subterminal loci of 35S rDNA (one in the larger and one in the smaller chromosome subset) and two loci of 5S rDNA, one localized interstitially in one of the larger chromosome pair, and a second subterminal in smaller chromosome pair (*M. linearilobum* marker), the same which carries also 35S rDNA (each rDNA type locus is localized in its different chromosome

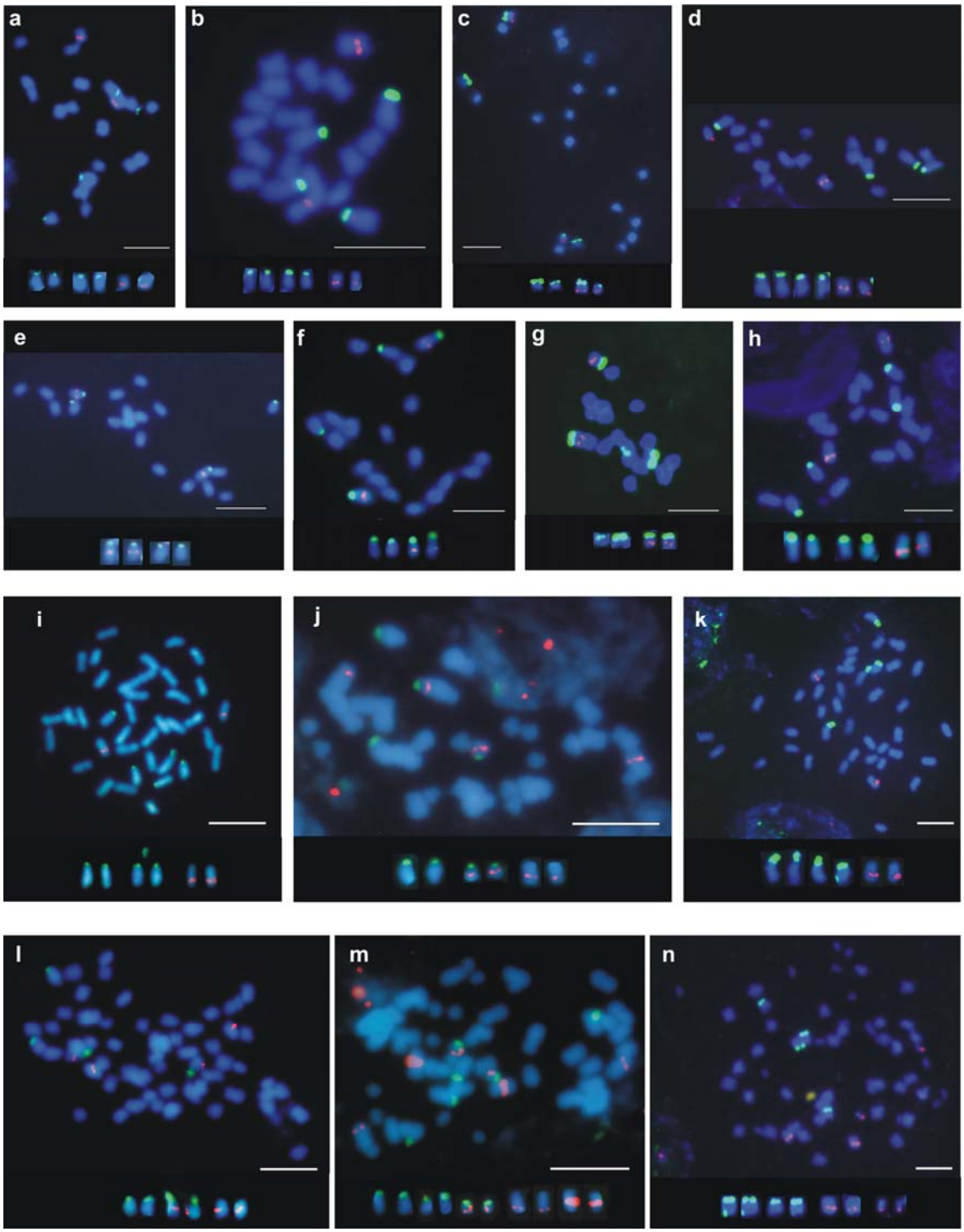


Fig. 4. *In situ* chromosomal localization of 5S (red signals) and 35S rDNA loci (green signals) in chromosomes of diploid (**a-h**), tetraploid (**i-k**) and hexaploid (**l-n**) species of sers. *Melampodium* (**a-d, i,**), *Cupulata* s.str. (**e-g**), *Glabribracteata* (**h**) and *Sericea* (**j-n**). **a,** *M. americanum* 19009, 2*n* = 2*x* = 20; **b,** *M. diffusum* 18671, 2*n* = 2*x* = 20; **c,** *M. linearilobum* 18665, 2*n* = 2*x* = 20; **d,** *M. pilosum* 18587, 2*n* = 2*x* = 20; **e,** *M. cupulatum* 19044, 2*n* = 2*x* = 20; **f,** *M. rosei* 19050, 2*n* = 2*x* = 20; **g,** *M. tenellum* 19022, 2*n* = 2*x* = 20; **h,** *M. glabribracteatum* 18654, 2*n* = 2*x* = 20; **i,** *M. mayfieldii* 19019, 2*n* = 4*x* = 40; **j,** *M. nayaritense* 1575, 2*n* = 4*x* = 40; **k,** *M. strigosum* 18728, 2*n* = 4*x* = 40; **l,** *M. longicorne* 18824, 2*n* = 6*x* = 60; **m**, *M. pringlei* 18637, 2*n* = 6*x* = 60; **n,** *M. sericeum* 18620, 2*n* = 6*x* = 60; scale bar = 5μm.

arm). This species has lost two parental loci of 35S rDNA, one maternal (*M. linearilobum*) and one paternal (*M. longipes*/*M. americanum*). Tetraploid *M. mayfieldii* (Fig. 4i) carries two pairs of 35S rDNA loci and one pair of 5S rDNA, all in different chromosomes. This tetraploid lost one 5S rDNA locus from the maternal parent (diploid of ser. *Melampodium*) and two 35S rDNA loci, at least one of which originated from the maternal parent. Hexaploid *M. longicorne* (Fig. 4l) possesses two pairs of 35S rDNA loci, both located subterminally, and two loci of 5S rDNA, one in interstitial position within independent chromosome, and another one interstitially within the other arm of one of the chromosomes carrying 35S rDNA loci (chromosomal marker of ser. *Cupulata*). It has lost two parental loci of 35S rDNA (at least one of maternal *M. strigosum* origin). Hexaploid *M. pringlei* (Fig. 4m) carries three loci of 35S rDNA loci and three loci of 5S rDNA. All loci are located on different chromosomes, except for one each of 35S and 5S rDNA loci that are localized on the same chromosome although on its different arms (*M. linearilobum* marker). This genome lost one parental locus of 35S rDNA (of paternal *M. linearilobum* origin). All remaining 35S rDNA repeats in *M. pringlei* have been converted to maternal parent type repeat (*M. linearilobum*), regardless of their parental origin. *Melampodium pringlei* is the only analysed polyploid in which an additional 5S rDNA locus has been found surpassing the number of 5S rDNA loci inheritend from parental taxa. *Melampodium sericeum* (Fig. 4n) has two loci of 35S rDNA (both from the larger chromosomal set), and two loci of 5S rDNA (one on the big and one on the small chromosome pair) all localized in different chromosomes. Two paternal 35S rDNA loci (of *M. linearilobum* origin) were lost from this genome.

Genome size – Genome size of the diploid species of series *Cupulata* s.str. and *Melampodium* varies significantly (Table 2). Near complete uniformity of genome size within the series has been recorded for species of ser. *Cupulata* s.str. (1C 0.91-1.00 pg), but some variation has been seen in ser. *Melampodium* p.p. (excluding *M. linearilobum*)

with two value classes present (1C, 1.03-1.05 pg and 1.11-1.15 pg). The species of ser. *Cupulata* s.str. in general have lower genome sizes compared to those of diploid species of ser. *Melampodium*. Two taxa have significantly different genome size values: *M. glabribracteatum* (ser. *Glabribracteata*) has a much higher genome size (c. 100%; 1C 1.85 pg) than the species of ser. *Cupulata* s.str. where it has previously been placed; and *M. linearilobum* has significantly lower genome size (c. 55%; 1C 0.49 pg) than the rest of species in ser. *Melampodium*. The same two species have also significantly different karyotype lengths. Out of six analysed polyploid species, five exhibit complete (*M. strigosum* and *M. longicorne*) or near-complete (*M. nayaritense*, *M. pringlei*, and *M. sericeum*) additive genome size values compared to inferred diploid progenitors. *Melampodium mayfieldii* is the only polyploid species with a genome size that is considerably higher (11%) than the expected additive value. Species forming cohesive phylogenetic lineages (Blöch *et al.*, 2009; Table 2) have very similar C-values. In most cases genome size of the allopolyploids was slightly lower than expected, but these differences have not been tested in mutiple accessions.

Discussion

Origin of the allopolyploids - Polyploidy is widely accepted to contribute to plant speciation, evolution and diversification (Otto & Whitton, 2000; Wendel, 2000; Rieseberg & Willis, 2007; AR Leitch & IJ Leitch, 2008; Wood *et al.*, 2009). In contrast to autopolyploids, which are often morphologically indistinguishable from their diploids progenitors (DE Soltis *et al.*, 2007), both homoploid diploid hybrids and allopolyploids are relatively well differentiated from their progenitors and often confer rapid reproductive isolation from parental taxa (Rieseberg & Willis, 2007). Hence, allopolyploidy has been studied more extensively in plants, and the origin of several diploid-allopolyploid systems has been well documented (e.g., Sang *et al.*, 1995;

Hughes *et al.*, 2002; Rieseberg & Willis, 2007; Kim *et al.*, 2008; Slotte *et al.*, 2008; Ainouche *et al.*, 2009; Paun *et al.*, 2009).

Recently, the use of single/low copy nuclear markers has enabled more detailed analyses and hypothesis-testing concerning origin of allopolyploids in several plant groups (reviews of Sang, 2002, and Small *et al.*, 2004; *Paeonia*, Ferguson & Sang, 2001; *Persicaria*, Kim *et al.*, 2008; *Cardamine asarifolia*, Lihová *et al.*, 2006; *Arabidopsis kamchatica*, Shimizu-Inatsugi *et al.*, 2009). These genomic regions are biparentally inherited (in contrast to plastid markers) and less susceptible to genomic turnover as compared to nuclear ITS sequences (homogenization, conversion etc.; Álvarez & Wendel, 2003; Poczai & Hyvönen, 2009). The use of two paralogues of low copy gene *Pgi*C and of repetitive 5S rDNA spacer (rarely suffering from homogenization) has proven valuable in unravelling the origin and pinpointing putative parental taxa of at least three putative allopolyploids of *Melampodium* (*M. nayaritense*, *M. pringlei*, and *M. strigosum*) for which other data were inconclusive, and provided deeper insight into the origin of other polyploids.

Some of the polyploids of *Melampodium* have previously been speculated to be of hybrid origin, based on their intermediate morphology (e.g., *M. paniculatum*, sect. *Zarabellia,* Stuessy & Brunken, 1979; *M. argophyllum*, sect. *Melampodium*, ser. *Leucantha*, Stuessy *et al.*, 2004, now shown to be rather of autopolyploid origin, C. Rebernig *et al*., in prep.), incongruent results in plastid and nuclear markers (*M. longicorne*, *M. mayfieldii*¸ *M. pringlei*, Blöch *et al.*, 2009), or bimodal karyotypes (*M. nayaritense* and *M. pringlei*, Weiss-Schneeweiss *et al.*, 2009; *M. sericeum*, Stuessy, 1970; Weiss-Schneeweiss *et al.*, 2009). Allopolyploid origin has now been unambiguously confirmed for all six here analysed polyploids of sect. *Melampodium*. The new data allow identification of putative parental taxa for tetraploid *M. strigosum*, which has now also been shown to be involved as the maternal parent in the origin of

the three hexaploid species: *Melampodium pringlei* and *M. sericeum* (together with *M. linearilobum*), and *M. longicorne* (together with one of the species of ser. *Cupulata* s.str.). *Melampodium strigosum* has earlier been hypothesized to be the donor of the 20 larger chromosome pairs of *M. sericeum* (Stuessy, 1970) and to be involved in hybridization and intogression events with *M. longicorne* in southeastern Arizona (Stuessy, 1968, 1971). A possible second parental taxon (and donor of the 10 small chromosome pairs) of *M. sericeum* was suggested to be *M. nayaritense* (Stuessy, 1970) at that time chromosomally unknown. This hypothesis has its merits as tetraploid *M. nayaritense* is a hybrid between *M. linearilobum* and a species close to *M. longipes*/*M. americanum*, and thus shares two of the three haploid genomes of *M. sericeum* and *M. pringlei*. An allopolyploid origin of the tetraploid *M. nayaritense* has now been shown for the first time and is at least partly concordant with earlier observations of Stuessy (1979) and Stuessy & Crisci (1984) who recognized *M. nayaritense* to be a morphological intermediate between ser. *Sericea*, where it was placed originally (Stuessy, 1972), and ser. *Melampodium* to which it tied both in cladistic as well as in phenetic analyses. Pinpointing putative parental taxa (or the progenitors thereof) has been more difficult for the two interserial polyploid hybrids, *M. longicorne* and *M. mayfieldii*. Phylogenetic signal allowed unequivocal identification of only one parental taxon for *M. longicorne* (tetraploid *M. strigosum*), whereas the other parent remains unclear (perhaps one of the extant species of series *Cupulata* s.str., possibly *M. appendiculatum*). However, no putative parental taxa could be unequivocally identified for the second interserial hybrid *M. mayfieldii*, beyond confirming its hybrid origin involving some taxa of sers. *Melampodium* and *Cupulata* s.str. *Melampodium diffusum* and *M. tenellum* could be suggested as most likely parents, but with low support. A similar situation was found in other allopolyploid species groups, e.g., in peonies, where only one parent of the allotetraploid *Peonia officinalis* was identified as *P. peregrina*

with the other parent as one member of the *P. arietina* species group (Ferguson & Sang, 2001).

Habitat and distribution range of diploids and allopolyploids – The distribution range of the analysed polyploids of *Melampodium* and their putative parental species/series (Fig. 5; Stuessy, 1972; Sundberg & Stuessy, 1990) is at least partly overlapping. This is true for the widespread allotetraploid *M. strigosum* and its two putative parents *M. americanum* and *M. glabribracteatum* (the latter being restricted to the type locality in Oaxaca, Mexico). Another widespread diploid species, *M. linearilobum* (ser. *Melampodium*), involved in three allopolyploid origin events, overlaps with *M. longipes* (possibly forming the allotetraploid *M. nayaritense*) and with *M. strigosum* (giving rise to the two allohexaploids *M. pringlei* and *M. sericeum*). The interserial allotetraploid *M. mayfieldii* is known only from the type locality near El Tuito, Jalisco, Mexico, where two putative suggested parental species (*M. diffusum* in Colima and *M. tenellum* of ser. *Cupulata* s.str. up to Michoácan) partly overlap in their distribution. Allohexaploid *M. longicorne* occurs between Sonora/Chihuahua (Mexico) and Arizona (USA) where some species of ser. *Cupulata* s.str., e.g. *M. appendiculatum*, meet with the maternal parent, allotetraploid *M. strigosum* (ser. *Sericea*).

The polyploids and their respective putative parents also share at least some ecological/vegetation characteristics. All five polyploids of ser. *Sericea*, as well as the only polyploid species of ser. *Melampodium*, *M. mayfieldii*, are pine-oak forest species, similarly to the diploids *M. glabribracteatum* (ser. *Glabribracteata*), *M. appendiculatum* (ser. *Cupulata* s.str.), *M. linearilobum*, *M. americanum*, *M. longipes*, and *M. pilosum* (ser. *Melampodium*). Only two polyploids, tetraploid *M. strigosum* and its hexaploid offspring *M. sericeum*, have been successful in spreading and colonizing new areas beyond the current distribution range of their parental taxa. All other polyploids are restricted to much smaller areas**.**

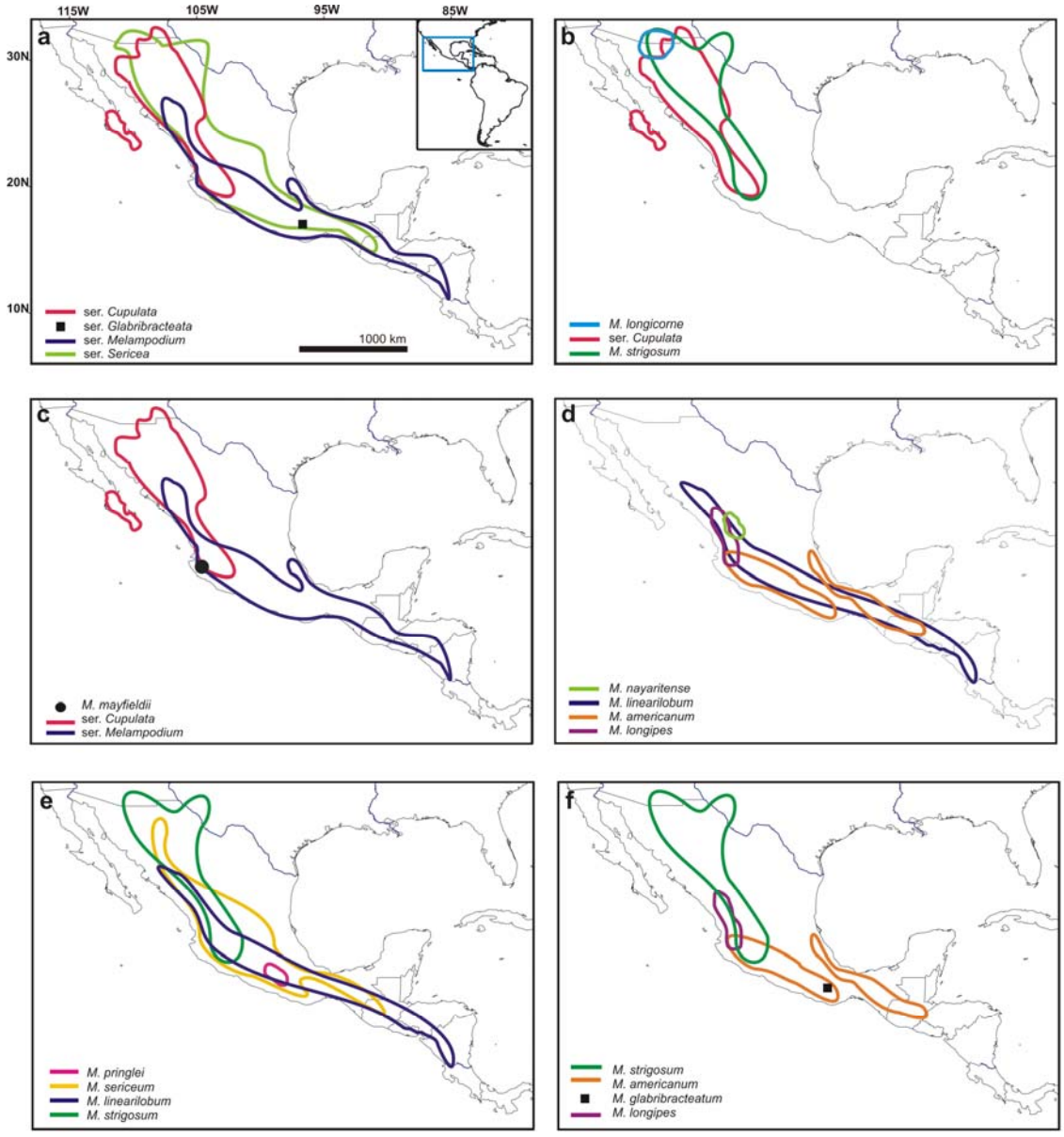


Fig. 5. Distribution maps of series in *Melampodium* sect. *Melampodium* plus analysed allopolyploids and their putative parental taxa. **a,** sers. *Melampodium*, *Cupulata* s.str., *Glabribracteata***,** *Sericea*; **b,** *M. longicorne* (6*x*, ser. *Sericea*), *M. strigosum* (ser. *Sericea*), ser. *Cupulata* s.str.; **c,** *M. mayfieldii* (4*x*,ser. *Melampodium*), ser. *Cupulata* s.str., ser. *Melampodium*; **d,** *M. nayaritense* (4*x*,ser. *Sericea*), *M. longipes* and *M. americanum*, *M. linearilobum* (ser. *Melampodium*); **e,** *M. pringlei* and *M. sericeum* (both 6*x*, ser. *Sericea*), *M. strigosum* (ser. *Sericea*), *M. linearilobum* (ser. *Melampodium*); **f,** *M. strigosum* (4*x*,ser. *Sericea*), *M. longipes* and *M. americanum* (ser. *Melampodium*), *M. glabribracteatum* (ser. *Glabribracteata*).

Genome diploidization and chromosomal rearrangements in the allopolyploids - Several groups of plants with well-documented allopolyploid history have recently been subjected to in-depth analyses of genomic, karyotypic and epigenomic evolution (*Senecio*, Ashton & Abbott, 1992; Abbott & Lowe, 2004; *Brassica*, Song *et al.*, 1995; Gaeta *et al.*, 2007; *Gossypium*, Wendel *et al.*, 1995; Adams

et al., 2009; *Spartina*, Baumel *et al.*, 2002; *Tragopogon*, DE Soltis *et al.*, 2004; Lim *et al.*, 2008; *Nicotiana*, Kovařík *et al.*, 2008; IJ Leitch *et al.*, 2008). Although all these studies have proven that hybridization and/or polyploidy are strong stimulants of genomic changes, the type, timing and the extent of these changes were shown to vary depending on the system studied (for review see, e.g., Hufton & Panopoulou, 2009). Genome rearrangements can be either directional or random and may occur rapidly as early as in the first few generations of the hybrids (*Brassica*, Song *et al.*, 1995; Gaeta *et al.*, 2007), or may be very minor and slow (*Spartina*, Yannic *et al.*, 2004). Inter- and intragenomic chromosomal rearrangements in polyploids are likely aimed at allowing homologous/faithful chromosome pairing in meiosis resulting in elevated recombination rates, and eventually functional genome diploidization (Nicolas *et al*., 2008; Cifuentes *et al*., 2009; Le Comber *et al.*, 2010).

Most readily detected chromosomal changes in plants are associated with localization of the abundance of various repetitive DNA types. Detailed comparative cytogenetic analyses of non-model groups of plants are limited by the availability of suitable chromosomal markers (probes), since the isolation of species/genus-specific tandem repeats has until now been time and labour consuming. The first-choice chromosomal markers applied to study non-model plant groups are the two types of conserved housekeeping rRNA genes (5S rDNA and 35S rDNA) present in at least one locus per any individual (Małuszyńska *et al.*, 1998). These two types of rDNA (usually) evolve independently in the genome. 35S rDNA is regularly subjected to DNA homogenization/conversion and activity changes of individual loci, while 5S rDNA is not commonly known to experience such phenomena (Fulneček *et al.*, 2002). Comparative analyses of rDNA loci localization in chromosomes combined with ITS (35S rDNA) and 5S rDNA NTS sequence analyses provide insight into the processes governing the evolution of the individual loci in their genomes. Several general trends

in evolution of rDNA in analysed polyploids of *Melampodium* are apparent (Fig. 6). 35S rDNA evolution involved in all polyploids: (1) loss of one or two parental 35S rDNA loci, and (2) conversion of the remaining 35S rDNA loci stochastically towards either maternal or paternal parent-type sequences (in disagreement with nuclear-cytoplasmic interaction theory; Lim *et al.*, 2005). 5S rDNA loci have all been retained in all polyploid genomes with the exception of *M. strigosum* (loss of paternal locus) and *M. mayfieldii* (loss of maternal locus originating from ser. *Melampodium*). Only one polyploid, *M. pringlei*, has gained one additional locus of 5S rDNA surpassing the sum of the loci seen in the parental taxa. Two possible hypothesis can be evoked to explain such a pattern: (1) independent origin of *M. pringlei* and *M. sericeum* from the same maternal and paternal taxa, involving different genotypes of the parental taxa that could additionally vary in 5S rDNA loci number; (2) gain of additional 5S rDNA locus in *M. pringlei* after the polyploid establishment and separation from its sister taxon *M. sericeum* (see also below). 5S rDNA loci homogenization has not been observed in any of the allopolyploids.

The parental genomes of three of the *Melampodium* allopolyploids, *M. nayaritense*, *M. pringlei* and *M. sericeum*, significantly differ in genome/chromosome size and can easily be distinguished in the polyploid nuclei, even without aid of GISH (proven to be rather ineffective in *Melampodium*; H. Weiss-Schneeweiss, unpubl.). *Melampodium linearilobum*-type of 35S rDNA parental repeats seems to be dominant over the other-parent copies when retained in the polyploid genome (in *M. nayaritense* and *M. pringlei*), regardless if *M. linearilobum* has acted as the maternal or paternal parent and triggered complete loci conversion. This pattern is maintained even if only one of two *linearilobum*-type 35S rDNA loci has been retained in the polyploid (and is in the minority), as seen in both *M. nayaritense* and *M. pringlei*. *Melampodium sericeum*, although the origin of this species is identical to *M. pringlei*, has lost both of

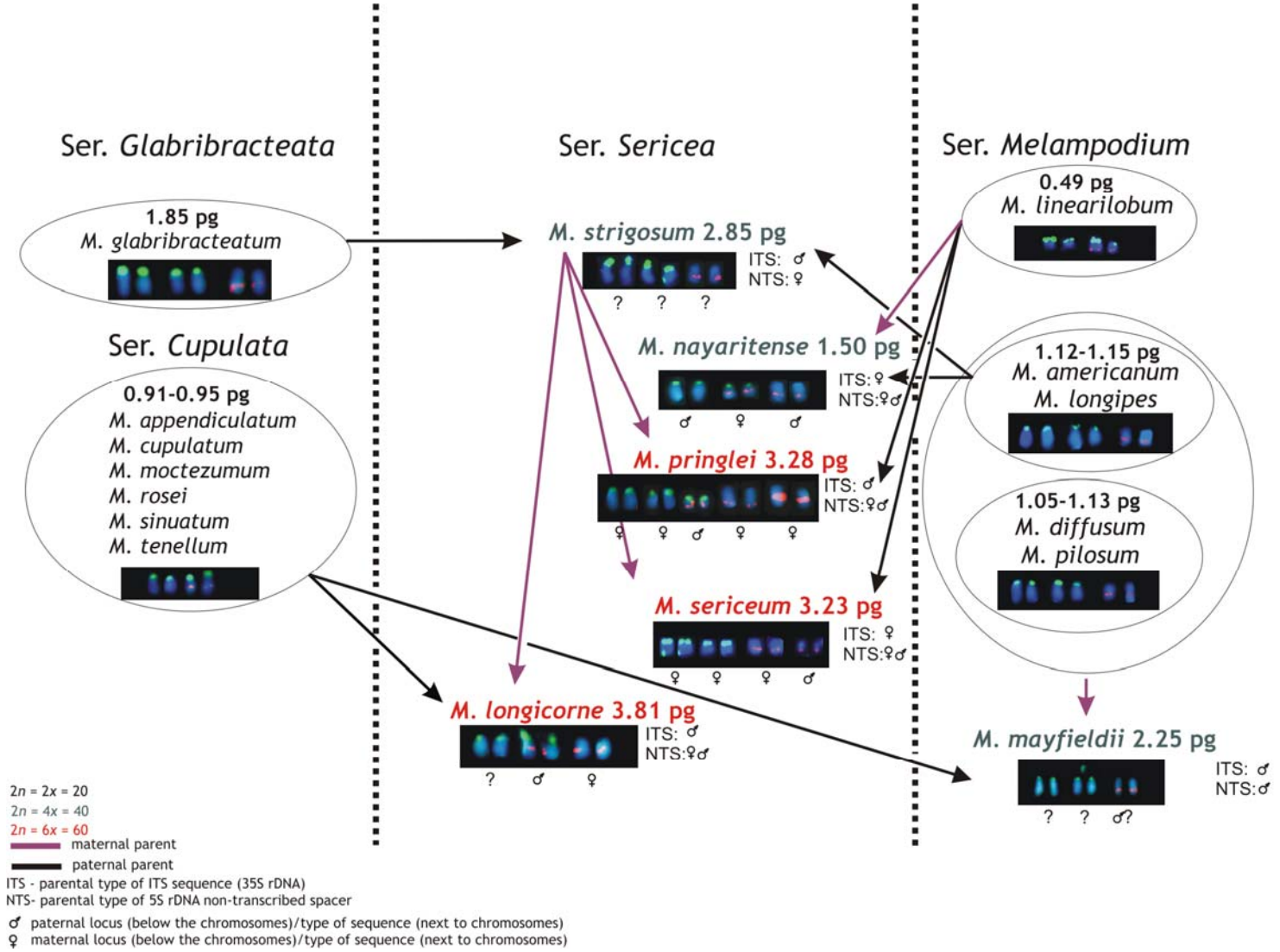


Fig. 6. Schematic representation of relationships between species and origin of polyploids. Indicated in this scheme are paternal (violett lines) and maternal (black lines) parents of the allopolyploids, ploidy level, FISH signals (with reference to paternal or maternal origin, where known), genome size, type of ITS and 5S rDNA NTS sequence.

the *M. linearilobum* loci. Such a tendency toward one-parent rDNA loci removal/modification in polyploid genomes has been reported in several plant groups, e.g., *Scilla autumnalis* (Vaughan *et al.*, 1993), *Tragopogon* (Lim *et al.*, 2008), or *Nicotiana* (Kovařík *et al.*, 2008). The change of rDNA loci number or localization has been shown in several other non-model allopolyploid species groups (e.g., Vaughan *et al.*, 1993; Lim *et al.*, 2000; Weiss & Małuszyńska, 2000; Weiss-Schneeweiss *et al.*, 2007, 2008; Kovařík *et al.*, 2008), more often involving 35S rDNA than 5S rDNA (Fulneček *et al.*, 2002).

The extent and rate of chromosomal and genomic changes in polyploids has thus been shown to correspond roughly to the age of the polyploids at least in some analysed plant groups ("genome turnover"; Lim *et al.*, 2005), ranging from additive parental (rDNA) loci number and localization in very young polyploids (e.g., *Tragopogon*, Pires *et al.*, 2004; *Spartina anglica*, Fortune *et al.*, 2007) to extreme cases of fully (secondarily) diploidized old paleopolyploids such as, e.g., maize (Gaut & Doebley, 1997) or *Arabidopsis thaliana* (Bowers *et al.*, 2003; Lysak *et al.*, 2006). However, even in young polyploids the basic repeats constituting the individual loci may be subjected to DNA homogenization/conversion and epigenetic changes (e.g., *Nicotiana*, Kovařík *et al.*, 2008; *Tragopogon*, Lim *et al.*, 2008). Although no dating of the *Melampodium* polyploids has been done other than general degree of sequence divergence, tetraploid *M. strigosum* is likely older than any of its three offspring allohexaploids. The tetraploid exhibits also more chromosomal changes (both types of rDNA loci loss) and nearly complete rDNA loci diploidization. Similarly, tetraploid *M. mayfieldii* is also likely an older polyploid, and also carries the signature of a diploidized genome (as judged from rDNA evolution).

Genome size evolution - The rather dynamic and mosaic changes of rDNA loci in polyploids remain in contrast to their stable and additive genome size values and

suggest the presence of well-balanced processes reshaping the genomes. The size of genomes of the hybrids, particularly allopolyploids, often experiences significant downsizing in comparison to the sum of parental genomes participating in their formation (e.g., Weiss-Schneeweiss *et al.*, 2006; AR Leitch & IJ Leitch, 2008; IJ Leitch *et al.*, 2008), less frequently increase and sometimes remaining similar to the sum of the parental genome sizes (Bennetzen *et al.*, 2005; IJ Leitch *et al.*, 2008). These changes strictly depend on the dynamics of the processes governing amplification of repetitive DNA elements in the genome and counterbalancing processes of their removal, but are also a function of time that has elapsed since polyploid formation (e.g., Bennetzen *et al.*, 2005; IJ Leitch *et al.*, 2008). Although the age of polyploids might not be directly correlated with the direction of genome size change, increasing age correlates with increase in the amount of genome size change (IJ Leitch *et al.*, 2008).

Genome sizes of allopolyploid *Melampodium* species are surprisingly stable when compared to the expected additive values of the genome sizes of putative parental taxa. Polyploids have obviously undergone neither significant reduction nor expansion of the genomes. The analyses of rDNA dynamics in the genomes, regardless of their age (inferred from sequence divergence), suggests that the evolution of parental genomes in the allopolyploids is balanced, and the processes of amplification of new sequence types have likely been counterbalanced by processes of genomic deletions. The only allopolyploid species that shows higher genome size value than expected is tetraploid *M. mayfieldii*. Genome size additivity seems to be retained in most polyploids regardless of their age as seen in, e.g., tetraploid *M. strigosum* and its three offspring allohexaploids.

Independent origins versus allohexaploid divergence of *M. sericeum* and *M. pringlei* - Recurrent polyploidization and multiple origin of polyploids have been shown to occur frequently and commonly (e.g. Ashton & Abbott, 1992; DE Soltis & PS Soltis,

1999; Sharbel & Mitchell-Olds, 2001; DE Soltis *et al.*, 2004, 2009a,b; Yang *et al.*, 2006; Tate *et al.,* 2009), although single/local hybridization events have also been documented (Kochert *et al.*, 1996**;** Baumel *et al.*, 2001; Sall *et al.*, 2003; Ainouche, *et al*., 2009). In most cases recurrent polyploidization and/or hybridization events result in genetically similar allopolyploid taxa/populations (e.g., *Draba norvegica,* Brochmann & Elven, 1992; *Tragopogon miscellus* and *T. mirrus*, DE Soltis *et al.*, 2004) or autopolyploid cytotypes in diploid-polyploid complexes (*Chrysanthemum indicum*, Yang *et al.*, 2006; *M. cinereum*, Rebernig *et al.*, 2010). Such patterns suggest that evolution on a larger scale may repeat itself in independent lineages, and that genomic and epigenetic responses to polyploidization and hybridization may at least partly be pre-programmed (e.g., *Tragopogon*, DE Soltis *et al.*, 2009b). It is relatively rare that recurrent polyploidization events can lead to the formation of cryptic species. In *Spartina*, e.g., independent hybridization of *S. maritima* and *S. alterniflora* (with the same maternal and paternal parents) led to the formation of two hybrids: *S.* x *neyrautii* and *S.* x *townsendii* (Ainouche *et al.*, 2009). Both homoploid hybrids deviated from parental genome structural additivity, albeit exhibiting different patterns of transposable genome alterations. Hybridization between the same parental species in the genus *Helianthus* produced via independent hybridization events three homoploid diploid hybrid species adapted to different ecological conditions (Rieseberg *et al.*, 1990, Rieseberg, 1991, Gross *et al.*, 2003). Some of the recurrently formed allopolyploid species of *Glycine* subgenus *Glycine* tend to form distinct lineages (JJ Doyle *et al.*, 2004), similar to two arctic narrow endemics of *Saxifraga*, which despite having the same parentage, are morphologically differentiated into *S. svalbardensis* and *S. opdalensis* (Brochmann *et al.*, 2004). It is often impossible to distinguish between common hybrid/allopolyploid origin followed by divergent speciation vs. recurrent

origin from different genotypes of the same set of parental taxa and their independent genomic evolution.

Very few well documented cases of allopolyploid species diversification and further speciation exist such as, e.g., polyploids in *Gossypium* (reviewed in Adams & Wendel, 2004), where ancient hybridization of species with A-genome and species with a G-genome and subsequent radiation led to five different allopolyploid species. Similarly, polyploids of *Nicotiana* sect. *Repandae* likely resulted from hybridization (ca. 4.5 Myr ago) and subsequent diversification (Clarkson *et al*., 2005) from common progenitor.

Two hexaploid species of *Melampodium*, *M. pringlei* and *M. sericeum*, share the same ancestry with *M. strigosum* acting as maternal and *M. linearilobum* as paternal parents. It is not known, however, if they originated by recurrent hybridization leading independently to two differnt species, or had a single origin followed by diversification and speciation. The two species are morphologically similar, with *M. sericeum* having taller heads and outer involucral bracts, more ray florets and yellow-tipped paleae, whereas the latter are purple in *M. pringlei* (Stuessy, 1972).

The role of hybrid speciation in section *Melampodium* - *Melampodium* comprises 40 species (Stuessy, 1972; Turner, 1988, 1993, 2007) with several basic chromosome numbers (x = 9, 10, 11, 12, 14; Weiss-Schneeweiss *et al.*, 2009). Phenological isolation is not common within *Melampodium* as most species flower between August and September (Sundberg & Stuessy, 1990). Species with the same chromosome number are mostly isolated geographically, and species ocurring sympatrically usually have different chromosome numbers or ploidy levels (Sundberg & Stuessy, 1990). Allopatric distributions play an important role in the isolation of closely related species that share the same basic chromosome number (reviewed in Coyne & Orr, 2004). Sundberg & Stuessy (1990) indicated that some of the polyploids

of ser. *Sericea* and diploids of ser. *Melampodium* (*M. americanum*, *M. linearilobum*, and *M. longipes*) species had partly overlapping distributions (e.g., *M. strigosum* with *M. sericeum* or *M. longicorne*; *M. americanum*, *M. linearilobum*, and *M. longipes* with *M. sericeum*) and hypothesized that these species may be isolated largely by differing ploidy levels (4*x* vs. 6*x*; 2*x* vs. 6*x*). In the rare contact zones of species with the same chromosome number morphological intermediates have sometimes been observed, suggesting possible occurrence of sporadic hybridization (Sundberg & Stuessy, 1990).

It has recently become clear that hybridization not only occurs in *Melampodium*, but that at least 11 out of 40 species in the genus are well documented hybrids (Blöch *et al.*, 2009; Weiss-Schneeweiss *et al.*, 2009). Importantly, most evolutionarily successful hybridization events in *Melampodium* (10 out of 11) have been accompanied/caused by genome doubling (allopolyploidy; Blöch *et al.*, 2009). Current data, however, indicate possible common and ongoing hybridization among diploid species within sers. *Cupulata* and *Melampodium*, as suggested by the presence of divergent homeologues of the two low copy paralogues of the *Pgi*C gene. Regardless of its extent, however, clearly some hybridizing species are more successful than others, and these most notably include diploid *M. linearilobum* and tetraploid *M. strigosum*, each being a parent to three polyploids (two of these shared). Darlington (1937) proposed that closely related species may more likely produce homoploid hybrids and highly divergent diploids may more likely produce polyploids. This hypothesis has since been tested in several systems (Grant, 1981) and recently revisited using comparative analyses of genetic distances/phylogenetic divergence between diploids and their descendant polyploids (Chapman & Burke, 2007; Buggs *et al.*, 2009; Paun *et al.*, 2009). While all these studies indicate that homoploid hybrid formation tends to occur among closely related taxa, polyploid formation has been inferred as either preferentially occurring between divergent taxa (Paun et al., 2009), or corresponding to a random hybridization

pattern (Buggs et al., 2009). All analysed *Melampodium* allopolyploids involve relatively divergent parental species (e.g., no polyploids within the rather cohesive ser. *Cupulata*). Grant (1981) suggested also that some genotypes (within the species) may be more predisposed to produce polyploids than others. Although some evidence suggests that parental genotypes might influence the likelihood of polyploid emergence (e.g., *Tragopogon*, Tate *et al*., 2009), this hypothesis has yet to be tested.

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CONCLUSIONS

The genus *Melampodium* encompasses a moderate number of species (currently 40) for which a recent taxonomic treatment is at hand (Stuessy, 1972, 1979; Stuessy & Crisci, 1984). Furthermore detailed chromosomal data revealed a multitude of haploid numbers (based on five basic numbers and high frequency of polyploids; Turner & King, 1961; Stuessy, 1971; Weiss-Schneeweiss & al., 2009). Therefore the genus is an ideal group in which to test the predictive value of chromosome numbers for phylogenetic relationships, and to study chromosome number evolution both on diploid and polyploid levels, as well as hybrid speciation.

Analyses of selected plastid and nuclear markers allowed elucidation of phylogenetic relationships within *Melampodium* and testing and refining the latest classification (Stuessy, 1972). *Melampodium* was identified as a paraphyletic genus with the genera *Acanthospermum* and *Lecocarpus* incorporated within it, but at ambiguous positions. Three of the six currently accepted sections of the genus (Stuessy, 1972) were supported to be monophyletic in phylogenetic analyses of both plastid *mat*K and nrITS, and five sections by the plastid marker alone. One of the currently recognized sections, sect. *Alcina* ($x = 11$), was shown to be polyphyletic in both marker sets. Plastid and nuclear phylogenies showed incongruencies mainly within sections *Melampodium*, *Zarabellia* and *Serratura*, giving strong indication for hybrid speciation in the evolution of these groups. Basic chromosome numbers were inferred to mostly correlate with major branches of the phylogeny. Currently these new insights are being incorporated into a refined taxonomic treatment of the genus (T.F. Stuessy & al., in prep.).

Chromosome number evolution in *Melampodium* was shown to be a dynamic process involving polyploidization (both autopolyploidy and allopolyploidy, with the latter being more frequent), dysploid loss or gain, as well as aneuploid loss of

chromosomes in some polyploids (Weiss-Schneeweiss & al., 2009). For *Melampodium*, both plastid as well as nuclear data point to an ancestral basic number of $x = 11$ for the genus and not $x = 10$, as previously proposed (Turner & King, 1961; Stuessy, 1971). From this ancestral base all other basic chromosome numbers have developed. The chromosomal base numbers $x = 12$ and 14, which share a common ancestor, originated once within the evolution of *Melampodium*. Neither single nor recurrent origin of both of the chromosomal base numbers of $x = 9$ and 10 can be excluded due to conflicting or inconclusive evidence from nuclear and plastid data. The basic chromosome number $x = 11$ was inferred as a symplesiomorphy shared by different unrelated lineages. Dysploid loss prevailed over dysploid gain. Dysploid gain was nevertheless an important factor in the chromosome evolution of *Melampodium*, in contrast with previous general theories, which played down the role of dysploid gain in karyotype evolution (Goldblatt & Johnson, 1988; Goldblatt & Takei, 1997). Furthermore, $x = 10$ of sect. *Melampodium*, although very likely not the ancestral character state, seemed to be the evolutionarily most successful lineage encompassing more than half of the species of the genus. Hybrid speciation was shown to contribute to the species richness of genus *Melampodium* with 11 out of 40 species of hybrid origin, especially in sect. *Melampodium*, which alone encompasses six allopolyploid species from sers. *Sericea* and *Melampodium*. These allopolyploids originated from repeated cycles of hybridization involving species of sect. *Melampodium*, sers. *Cupulata* s.str., *Glabribracteata*, and *Melampodium*. The relative genome size additivity observed in all allopolyploids contrasts with 35S rDNA loci loss and conversion and, to a much lesser extent, with loss of 5S rDNA loci, suggesting well-balanced genome re-organization mechanisms. Two hexaploid species, *M. pringlei* and *M. sericeum*, although originating from the same set of taxa, have followed different genome restructuring pathways as judged from rDNA loci.

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ABSTRACT

The genus *Melampodium* (Asteraceae) is a group very well-suited to study the evolutionary consequences of chromosome number change and reticulation. The genus comprises a moderate number (40) of annual and perennial species classified into six sections. The genus is centered in tropical to subtropical Mexico, southwestern United States, Brazil and Colombia. Chromosome numbers and karyotypes are now known for all species except for the recently described species *Melampodium moctezumum*. The genus displays a wide range of haploid chromosome numbers (n = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33), one of the longest series in family Asteraceae. These haploid chromosome numbers can be allocated to five basic chromosome numbers (x = 9, 10, 11, 12, and 14). The current classification of the genus is based primarily on a combination of morphological characters and basic chromosome numbers.

The first chapter of the thesis presents a molecular phylogeny of the genus based on analyses of plastid and nuclear DNA markers, which is also used to test the current taxonomic classification. Results show that: (1) *Melampodium* is monophyletic if closely related genera *Acanthospermum* and *Lecocarpus* are included; (2) reticulation and hybridization events have repeatedly contributed to the evolution of *Melampodium* as inferred from incongruencies between plastid and nuclear phylogenies; (3) three of the six sections of the current classification are supported by both marker systems, with five out of the six sections supported by the plastid phylogeny alone; (4) section *Alcina* encompassing three species has been inferred as polyphyletic in both marker sets; and (5) basic chromosome numbers correlate (at least partly) with the phylogeny of the genus.

In the second chapter the directionality of the chromosome number change has been investigated using plastid *mat*K and nuclear ribosomal ITS phylogenies of the diploid taxa and applying maximum parsimony and maximum likelihood-based

reconstruction methods. All analyses support $x = 11$ as the most likely ancestral basic chromosome number for the genus. The basic chromosome number of $x = 10$, previously hypothesized to be ancestral for the genus, was reconstructed to originate once (plastid data) or twice (nuclear data). Similarly, the chromosomal base number of $x = 9$ has likely originated twice independently, but a single origin cannot be excluded. The chromosomal base numbers $x = 12$ and 14 have been shown to be derived from a common ancestor most likely based on $x = 11$. Descending dysploidy was shown to be more prevalent than ascending dysploidy.

The third chapter examines the hybridization events leading to the origin of six allopolyploid species of sers. *Sericea* and *Melampodium* (sect. *Melampodium*), and their subsequent genome evolution. A combined approach employed sequencing of plastid (*mat*K, *psb*A-*trn*H) and nuclear DNA regions (ITS, 5SrDNA spacer, low copy *Pgi*C gene), restriction pattern analyses of ITS, 5S and 35S rDNA mapping in the chromosomes using fluorescence *in situ* hybridization (FISH), and flow cytometry for genome size measurements. These allowed inferring the origins of allopolyploids and tracing genome restructuring following the polyploid establishment. Species of sers. *Melampodium*, *Cupulata* s.str., and ser. *Glabribracteata* were shown to be involved as parental taxa in hybridization events leading to the origin of six allopolyploid species. The genome size additivity observed in all polyploids contrasts with 35S rDNA loci loss and conversion and, albeit to lesser extent, with loss of 5S rDNA loci. Two allohexaploids, *Melampodium pringlei* and *M. sericeum*, despite originating from the same parental taxa, have been shown to follow different genome restructuring pathways.

ZUSAMMENFASSUNG

Die Gattung *Melampodium* eignet sich gut für die Untersuchung der evolutionären Folgen von Chromosomenzahländerungen und Hybridisierungen. *Melampodium* umfasst 40 einjährige und mehrjährige Arten in sechs Sektionen und ist vorwiegend in Mittelamerika beheimatet. Chromosomenzahlen sind für alle Arten mit Ausnahme einer erst vor Kurzem beschriebenen Art bekannt. Die Gattung umfasst eine der längsten Ketten an haploiden Chromosomenzahlen innerhalb der Asteraceaen (n = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33). Diese lassen sich auf fünf Basischromosomenzahlen zurückführen. Die derzeitige Klassifizierung der Gattung basiert auf einer Kombination von morphologischen Merkmalen und Chromosomenzahlen.

Im ersten Kapitel wird eine auf Plastiden- und nukleären ITS-Sequenzdaten basierende molekulare Phylogenie präsentiert und die Klassifikation der Gattung getestet. Die Resultate lassen sich wie folgt zusammenfassen: 1) Die Gattung *Melampodium* ist monophyletisch, wenn die beiden Gattungen *Acanthospermum* und *Lecocarpus* inkludiert werden; (2) Hybridisierungen und Retikulation haben zur Evolution der Gattung beigetragen, wie durch die fehlende Übereinstimmung von Plastiden- und Kernsequenzdaten angedeutet wird; (3) Drei der sechs Sektionen der Gattung *Melampodium* werden von beiden Markersystemen unterstützt und fünf durch den Plastidenstammbaum alleine; (4) Die Polyphylie der Sektion *Alcina* wird durch beide Markersysteme belegt; (5) Die fünf Basischromosomenzahlen der Gattung sind zumindest teilweise indikativ für die Phylogenie der Gattung.

Im zweiten Kapitel wird die Richtung der Chromosomenbasiszahländerungen (absteigend vs. aufsteigend) innerhalb der Gattung mit Hilfe von Plastiden- und Kernsequenzdaten unter Anwendung von Maximum Parsimony und Maximum Likelihood Characterstate-Reconstruction-Analysen untersucht. In allen Analysen wird

x = 11 als die wahrscheinlichste Urchromosomenzahl der Gattung rekonstruiert. Die Anzahl der Linien der Basischromosomenzahl x = 10, die früher als ursprüngliche Basischromosomenzahl der Gattung postuliert wurde, ist nicht eindeutig. Durch die abweichende Positionierung einer Art kann weder ein einziger Ursprung noch die Entstehung in zwei verschiedenen Linien ausgeschlossen werden. Ebenso kann die Anzahl der Ursprünge der Basischromosomenzahl x = 9 nicht eindeutig festgelegt werden (einzelner Ursprung und nachfolgende Diversifikation bzw. Hybridisierung vs. zwei unabhängig voneinander entstandene Linien). Weiters unterstützen alle Analysen einen gemeinsamen Vorfahren der Basischromosomenzahlen x = 12 und 14, der wahrscheinlich auf x = 11 basierte. Absteigende Dysploidie ist in der Evolution von *Melampodium* häufiger als aufsteigende.

Das dritte Kapitel behandelt Hybridisierungs- und Polyploidiserungsvorgänge von allopolyploiden Arten der Serien *Sericea* und *Melampodium* (Sektion *Melampodium*). Mit einem kombinierten Ansatz (Sequenzdaten: Plastiden: *mat*K, *psb*-A-*trn*H; Kern-DNS: ITS, 5S rDNS Spacer, Low-Copy-Gen *Pgi*C; Restriktionsmusteranalyse von ITS; rDNA-Lokalisierung; Genomgrößenmessung) wird der Ursprung der allopolyploiden Arten und die Genomreorganisation nach der der Polyploidisierung untersucht. Die Ergebnisse zeigen, dass Arten der Serien *Melampodium*, *Cupulata* s.str. und *Glabribracteata* als Elternarten beteiligt sind. Die Genomgrößen der allopolyploiden Hybridarten entsprechen relativ genau der Summe der Elternarten. Dies steht im Gegensatz zur beobachteten Reduktion beider rDNA-Loci, sowie zur kompletten Umwandlung der 35S-rDNA-Loci zum Typus eines Elternteils. Zwei allohexaploide Arten, *Melampodium sericeum* und *M. pringlei*, die die gleichen Elternarten teilen, zeigen verschiedenartige Genomreorganisationen.

APPENDIX: NEW CLASSIFICATION OF THE GENUS *MELAMPODIUM*

Here we present the new classification of the genus *Melampodium* based on the combination of morphological characters and molecular plastid (*mat*K, *psb*A-*trn*H) and nuclear (ITS, 5S rDNA spacer, *Pgi*C) phylogenies (T. F. Stuessy, H. Weiss-Schneeweiss, C. Blöch, J. Villaseñor, and C.A. Rebernig, in prep.), which is used for the second and the third chapter.

Previous classification (Stuessy, 1972; including species described by Turner, 1988, 1993, 2007)

Section *Melampodium* (*x* = 10)
Series *Melampodium*
1. *M. americanum* L.
2. *M. diffusum* Cass.
3. *M. pilosum* Stuessy
4. *M. longipes* (A.Gray) B.L.Rob.
5. *M. linearilobum* DC.
6. *M. mayfieldii* B.L. Turner

Series *Leucantha* Stuessy
7. *M. leucanthum* Torr. & A.Gray
8. *M. cinereum* DC.
   8a. var. *cinereum*
   8b. var. *hirtellum* Stuessy
   8c. var. *ramosissimum* (DC.) A.Gray
9. *M. argophyllum* (A.Gray ex B.L.Rob.) S.F.Blake

Series *Sericea* Stuessy
10. *M. sericeum* Lag.
11. *M. pringlei* B.L.Rob.
12. *M. strigosum* Stuessy
13. *M. longicorne* A.Gray
14. *M. nayaritense* Stuessy

Series *Cupulata* Stuessy
15. *M. cupulatum* A.Gray
16. *M. appendiculatum* B.L.Rob.
17. *M. sinuatum* Brandegee
18. *M. rosei* B.L.Rob.
19. *M. tenellum* Hook.f & Arn.
20. *M. glabribracteatum* Stuessy
21. *M. moctezumum* B.L.Turner

Series *Longipila* Stuessy
22. *M. longipilum* B.L.Rob.

Section *Zarabellia* (Cass.) DC. (*x* = 9)
23. *M. longifolium* Cerv. ex Cav.
24. *M. mimulifolium* B.L.Rob.
25. *M. gracile* Less.
26. *M. microcephalum* Less.
27. *M. paniculatum* Gardner

Section *Serratura* Stuessy (*x* = 12)
28. *M. divaricatum* (Rich. in Pers.) DC.
29. *M. costaricense* Stuessy
30. *M. dicoelocarpum* B.L.Rob.
31. *M. tepicense* B.L.Rob.
32. *M. sinaloense* Stuessy
33. *M. northingtonii* B.L.Turner

Section *Bibractiaria* Stuessy (*x* = 14)
34. *M. bibracteatum* S.Watson
35. *M. repens* Sessé & Moç

Section *Rhizomaria* Stuessy (*x* = 11)
36. *M. montanum* Benth.
   36a. var. *montanum*
   36b. var. *viridulum* Stuessy
37. *M. aureum* Brandegee

Section *Alcina* (Cav.) DC. (*x* = 11)
38. *M. perfoliatum* (Cav.) H.B.K.
39. *M. glabrum* S. Watson
40. *M. nutans* Stuessy

New classification (Stuessy & al., in prep.)

Section *Rhizomaria* Stuessy (*x* = 11)
1. *M. montanum* Benth.
 1a. var. *montanum*
 1b. var. *viridulum* Stuessy
2. *M. aureum* Brandegee

Section *Glabrata* Stuessy (*x* = 11)
3. *M. glabrum* S. Watson

Section *Zarabellia* (Cass.) DC. (*x* = 9)
Series *Zarabellia* Cass.
4. *M. longifolium* Cerv. ex Cav.
5. *M. mimulifolium* B.L.Rob.

Series *Tribracteata* Stuessy
6. *M. microcephalum* Less.
7. *M. gracile* Less.
8. *M. paniculatum* Gardner

Section *Alcina* (Cav.) DC. (*x* = 11)
9. *M. perfoliatum* (Cav.) H.B.K.

Section *Bibractiaria* Stuessy (*x* = 14)
10. *M. bibracteatum* S.Watson
11. *M. repens* Sessé & Moç

Section *Serratura* Stuessy (*x* = 12)
12. *M. divaricatum* (Rich. in Pers.) DC.
13. *M. costaricense* Stuessy
14. *M. northingtonii* B.L.Turner
15. *M. tepicense* B.L.Rob.
16. *M. sinaloense* Stuessy
17. *M. dicoelocarpum* B.L.Rob.

Section *Nutantes* Stuessy (*x* = 11)
18. *M. nutans* Stuessy

Section *Melampodium* (*x* = 10)
Series *Longipila* Stuessy
19. *M. longipilum* B.L.Rob.

Series *Cupulata* Stuessy
20. *M. cupulatum* A.Gray
21. *M. appendiculatum* B.L.Rob.
22. *M. moctezumum* B.L.Turner
23. *M. sinuatum* Brandegee
24. *M. rosei* B.L.Rob.
25. *M. tenellum* Hook.f & Arn.

Series *Leucantha* Stuessy
26. *M. leucanthum* Torr. & A.Gray
27. *M. cinereum* DC.
27a. var. *cinereum*
27b. var. *hirtellum* Stuessy
27c. var. *ramosissimum* (DC.) A.Gray
28. *M. argophyllum* (A.Gray ex B.L.Rob.) S.F.Blake

Series *Glabribracteata* Stuessy
29. *M. glabribracteatum* Stuessy

Series *Sericea* Stuessy
30. *M. sericeum* Lag.
31. *M. pringlei* B.L.Rob.
32. *M. strigosum* Stuessy
33. *M. nayaritense* Stuessy
34. *M. longicorne* A.Gray

Series *Melampodium*
35. *M. americanum* L.
36. *M. diffusum* Cass.
37. *M. pilosum* Stuessy
38. *M. longipes* (A.Gray) B.L.Rob.
39. *M. linearilobum* DC.
40. *M. mayfieldii* B.L.Turner

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CURRICULUM VITAE

Personal details

Name, title:	Cordula Mariam Blöch, Mag.
Date/place of birth:	19th September 1981 in Vienna, Austria
Address:	Rauchgasse 39/17, A-1120 Vienna, Austria
E-mail:	cordula.bloech@unet.univie.ac.at
Marital status:	married, one son (*2009)

Education

1987-1991	Elementary School in Vienna XI, Wilhelm-Kress-Platz 33
1991-1999	Secondary School in Vienna X, Ettenreichgasse 41-43
1999	School leaving certificate with distinction
1999-2005	Course of study in biology/ecology (2nd subject botany) at the University of Vienna
March 2004	Adoption of the diploma theses theme, supervisor: ao.Prof. Dr. M.R. Samuel
November 2005	Final degree with distinction
Jan. 2006-April 2010	PhD student in project "*Melampodium*", o.Prof. Dr. T.F. Stuessy
March 2007	PhD-Course "Phylogenetic Systematics and Alignments" University of Copenhagen, Denmark.

Field excursions

June 2002	Island Krk, Kroatia
May 2002	Slovenian Karst
July 2003	Tyrol, Austria (Mapping excursion with Prof. H. Niklfeld)
Feb.-March 2004	Ecuador
July 2004	Carinthia
April 2005	Andalusia, Spain
June 2005	Hohe Tauern, Austria
September 2006	Northern and Central Mexico (Collection trip)
May 2007	New Mexico & Texas, USA (Collection trip)

Publications

Blöch, C., Weiss-Schneeweiss, H., Schneeweiss, G.M., Barfuss, M.H.J., Rebernig, C.A., Villaseñor, J.L., Stuessy, T.F. 2009. Molecular phylogenetic analysis of nuclear and plastid DNA sequences support the important roles of dysploid and polyploid chromosome number changes as well as of reticulate evolution in the diversification of *Melampodium* (Millerieae, Asteraceae). *Molecular Phylogenetics and Evolution* 53: 220-233.

Blöch, C., Dickoré, W. B., Samuel. R., Stuessy, T.F. 2010. Molecular phylogeny of the Edelweiss (*Leontopodium*, Asteraceae-Gnaphalieae). *Edinburgh Journal of Botany* 67: 235-264.

Participation in conferences

- Oral presentation at the 11th Meeting of Austrian Botanists (3 - 5 September 2004, Vienna, Austria)
- Poster presentation at 17th International Botanical Congress (12 – 16 July 2005, Vienna, Austria)
- Oral presentation at the 5th Biennal Conference of the Systematic Organisation (22 – 26 August 2005, Cardiff, UK)
- Poster presentation at the Meeting of the International Compositae Alliance (3 – 8 July 2006, Barcelona, Spain)
- 9. Jahrestagung der Gesellschaft für Biologische Systematik (GfBS), Natural History Museum (20-23 February 2007, Vienna, Austria)
- Oral presentation at the Sixt Biennial Conference of the Systematics Association (28-31 August 2007, Edinburgh, UK).
- Oral presentation at the Systematics 2008, 10th Annual Meeting of the Gesellschaft für Biologische Systematik (7-11 April 2008, Göttingen, Germany)
- Poster presentation Annual Meeting of the Society for Molecular Biology and Evolution (5-8 June 2008, Barcelona, Spain)
- Oral presentation at the 13. Österreichisches Botanikertreffen (11-12 September 2008, University of Salzburg, Austria)

Invited talks

January 2006: Phylogenetic relationships of the Edelweiss (*Leontopodium alpinum*) and its relatives; Botanical Society of Croatia and Biological Society of Croatia.

Abstracts of contributions to conferences (*presenting author; §published abstract)

- Blöch, C.*, Samuel, R., Stuessy, T. F., Dickoré, W. B. 2004. Die Evolution von Edelweiß (*Leontopodium*) und seinen Verwandten. *11. Österreichisches Botaniker Treffen* - Vienna, Austria.
- Blöch, C.*, Samuel, R., Dickoré, B., Stuessy, T. F. 2005. Molecular phylogenetics of Edelweiss (*Leontopodium*, Asteraceae). *XVII International Botanical Congress* – Vienna, Austria. p. 410
- Blöch, C.*, Dickoré, B., Samuel, R., Stuessy, T. F. 2005. Molecular phylogenetics of Edelweiss (*Leontopodium alpinum* Cass. Asteraceae) and its relatives. *5th Biennal Conference of the Systematics Organisation* – Cardiff, UK. p. 37
- Blöch, C.*, Barfuss, M. H. J., Stuessy, T. F., Rebernig, C. A., Weiss-Schneeweiss, H., Villaseñor, J. L. 2006. Molecular phylogeny and chromosome numbers in *Melampodium* (Asteraceae; Heliantheae). *Meeting of the International Compositae Alliance* – Barcelona, Spain. p.91
- Rebernig, C. A.*, Stuessy, T. F., Kohl, G., Weiss-Schneeweiss, H., Villaseñor, J. L., Blöch, C. 2006. Phylogeography of the white-rayed complex of the genus *Melampodium* (Asteraceae, Heliantheae). *Meeting of the International Compositae Alliance* – Barcelona, Spain. p.115
- Rebernig, C. A.*, Kohl, G., Obermayer, R., Stuessy, T. F., Weiss-Schneeweiss, H., Blöch, C., Villaseñor, J. L. 2007. Phylogeography in the genus *Melampodium* – a case study. *Symposium of the Botanical Society of Scotland* - St. Andrews, Scotland.
- Stuessy T. F.*, Blöch C., Weiss-Schneeweiss H., Villaseñor J. L. 2007. Molecular phylogeny, classification, and chromosomal evolution of *Melampodium* (Asteraceae, Heliantheae). *Botany 2007*, Chicago, USA.
- Schneeweiss, H.*, Stuessy, T. F., Blöch, C., Villaseñor, J. L. 2007. Chromosome number evolution, diploid karyotypes diversification, and polyploid karyotypes origin

in *Melampodium* (Asteraceae). *16th International Chromosome Conference* - Amsterdam, The Netherlands.

- Blöch, C.*, Weiss-Schneeweiss, H., Barfuss, M., Stuessy, T., Rebernig, C., Villaseñor, J. L. 2007. Molecular phylogeny and chromosomal changes in *Melampodium*. *The Sixt Biennial Conference of the Systematics Association* - Edinburgh, UK.
- Rebernig, C. A.*, Kohl, G., Weiss-Schneeweiss, H., Blöch, C., Obermayer, R., Villaseñor, J. L., Stuessy, T. F. 2007. Phylogeography and evolution of diploid cytotypes of the white-rayed complex of *Melampodium* (Asteraceae). *The Sixt Biennial Conference of the Systematics Association* - Edinburgh, UK.
- Blöch, C.*, Weiss-Schneeweiss, H., Barfuss, M., Rebernig, C., Villaseñor, J. L., Stuessy, T. F. 2008. Phylogeny of the genus *Melampodium* and the development of the $x = 10$ line. *Systematics 2008* - Göttingen, Germany.
- Rebernig, C. A.*, Kohl, G., Weiss-Schneeweiss, H., Blöch, C., Obermayer, R., Villaseñor, J. L., Stuessy, T. F. 2008. Polyploidization and phylogeography in the three varieties of *Melampodium cinereum* (Heliantheae, Asteraceae). *Systematics 2008* - Göttingen, Germany.
- Blöch, C.*, Rebernig, C., Weiss-Schneeweiss, H., Barfuss, M., Villasenor, J. L., Stuessy, T. F. 2008: Phylogeny and chromosomal evolution of the genus *Melampodium*. *Annual Meeting of the Society for Molecular Biology and Evolution* – Barcelona, Spain.
- Rebernig, C. A.*, Weiss-Schneeweiss, H., Blöch, C., Obermayer, R., Villaseñor, J. L., Stuessy, T. F. 2008. *Melampodium cinereum* (Heliantheae, Asteraceae) – phylogeography and polyploidy. *Annual Meeting of the Society for Molecular Biology and Evolution* - Barcelona, Spain.
- §Blöch, C.*, Weiss-Schneeweiss, H., Barfuss, M. H. J., Rebernig, C. A., Villasenor, J. L., Stuessy, T.F. 2009. Phylogeny of the genus *Melampodium* (Heliantheae, Asteraceae) and the orgins of poylploids of sect. *Melampodium. 13. Österreichisches Botanikertreffen* – Salzburg, Austria; *Sauteria* 16, p. 178.
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- §Rebernig, C. A.*, Weiss-Schneeweiss, H., Blöch, C., Obermayer, R.., Villasenor, J. L Stuessy, T.F. 2009. Phylogeography and polyploidy in the white-rayed complex of the genus *Melampodium* (Asteraceae); *13. Österreichisches Botanikertreffen* – Salzburg, Austria; *Sauteria* 16, p. 251.
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