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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)

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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 (*matK*) 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; *matK*; *Melampodium*; Phylogeny; Polyploidy; Reticulate evolution

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1. Introduction

Chromosome evolution, involving both number (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* (Asteraceae). 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.): $n = 9, 10, 11, 12, 14, 18, 20, 23, 24, 27, 28, 30, 33$.

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; Eliasson, 1971; Adersen, 1980; Sönderberg Brok and Adersen, 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 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 *matK* 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 *matK* 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 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 *trnK* intron including the complete *matK* 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 MgCl₂ with the addition of 4% dimethyl sulfoxide (DMSO) for ITS or 0.02% bovine serum albumin (BSA) for *matK*. 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 (*matK*) 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 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 *matK*; 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 (*trnK* intron, the combined first and second codon position of the *matK* gene, the third codon position of the *matK* 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 1000th 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 *matK* 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 $2 \times \ln \text{BF}$, considering $2 \times \ln \text{BF}_{\text{model 1 vs. model 2}} > 10$ 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; Herskovitz 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 (Herskovitz 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 628 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 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 $-\ln = -8636.03$), 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 $2 \times \ln \text{BF}$ 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 $2 \times \ln \text{BF}$ 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 $2 \times \ln \text{BF}$ of -53.98 ; Table 4). The three species, which form single species clades with uncertain 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 ($2 \times \ln \text{BF}$ of nuclear -57.72 ; Table 4). Concluding so far, 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 glabribacteatum* 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).

3.2. matK

The data matrix comprised 90 accessions of Melampodiinae and one accession each of the outgroup taxa *Acmella*, *Galinsoga*, *Millieria* and *Smallanthus* (Table 1). Sequence length was 222–394 bp for the *trnK* intron and 1479–1530 bp for the *matK* 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 $-\ln = -7853.81$), 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 ($2 \times \ln BF$ 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 ($2 \times \ln BF$ 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 *matK* 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 and 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 and 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. *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; Böhm 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 and 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 and 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 and 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 *pgiC* sequences (C. Blösch 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 involucre (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 and 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 uncertainties 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 autopolyploidisation 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 and 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 and 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 *matK* 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. Blösch 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.

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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 *matK* 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).

Fig. 3.

Consensus network from 50 trees each of the set of posterior trees from the ITS and the *matK* 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 and 2) are also shown. Scale bar represents mean edge weights.

Table 1

Species names, localities, voucher numbers, ploidy levels (H. Weiss-Schneeweiss et al., unpubl.), 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. Blösch; CR, C.A. Rebernig; CSB, Camilla Sønderberg Brok, EO, E. Ortiz B.; GF, G. Flores; HA, H. Adersen; IC, I. Calzada; IS, I. Sánchez; JC, J. Calónico; JV, J.L. Villasenor; 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)	Accession	Collection details, voucher numbers	GenBank accession numbers	
			ITS	matK
Melampodiinae				
<i>Melampodium</i>				
Sect. <i>Melampodium</i> (x = 10)				
Ser. <i>Melampodium</i>				
<i>M. americanum</i> L. (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18592.	FJ696977	FJ697080
	2	M, Colima, 2005; TS, JV, CR & IC, 18609.	FJ696978, FJ696979	FJ697081
<i>M. diffusum</i> Cass. (2x)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18666.	FJ696975	FJ697082
	2	M, Guerrero, 2005; TS, JV, CR & IC, 18669.	FJ696976	FJ697083
<i>M. linearilobum</i> DC. (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18593.	FJ696983	FJ697088
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18661.	FJ696982	FJ697089
<i>M. longipes</i> (A. Gray) B.L. Rob. (2x)	1	M, Nayarit, 2005; TS, JV, CR & IC, 18619.	FJ696984	FJ697087
	2	M, Nayarit, 2005; TS, JV, CR & IC, 18621.	FJ696985	FJ697086
<i>M. mayfieldii</i> B.L. Turner (4x)	1	M, Colima, 2005; TS, JV, CR & IC, 18613.	FJ697018	FJ697112
	2	M, Jalisco, 2006; TS, JV, CB & EO, 19019.	FJ697019–FJ697021	FJ697113
<i>M. pilosum</i> Stuessy (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18587.	FJ696981	FJ697084
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18590.	FJ696980	FJ697085
Ser. <i>Leucantha</i>				
<i>M. argophyllum</i> (A. Gray ex B.L. Rob.)	1	M, Nuevo León, 2006; TS, JV, CR & CB, 19059.	FJ697009	FJ697110
S.F. Blake (6x)	2	M, Nuevo León, 2006; TS, JV, CR & CB, 19060.	FJ697010–FJ697013	FJ697111
<i>M. cinereum</i> DC. var. <i>cinereum</i> (2x, 4x)	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
<i>M. cinereum</i> DC. var. <i>hirtellum</i> Stuessy (2x)	1	M, Coahuila, 2006; TS, JV, CR & CB, 19057.	FJ697015	FJ697104
	2	M, Nuevo León, 2006; TS, JV, CR & CB, 19061.	FJ697014	FJ697105
<i>M. cinereum</i> DC. var. <i>ramosissimum</i> DC. (A. Gray) (2x)	1	M, Tamaulipas, 2006; TS, JV & CB, 19063.	FJ697016	FJ697106
	2	M, Tamaulipas, 2006; TS, JV & CB, 19064.	FJ697017	FJ697107
<i>M. leucanthum</i> Torr. & A. Gray (2x, 4x)	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. <i>Sericea</i>				
<i>M. longicorne</i> A. Gray (6x)	1	USA, Arizona, Pima Co, 2006; CR & MB, 18823.	FJ697000	FJ697098
	2	USA, Arizona, Pima Co, 2006; CR & MB, 18826.	FJ697001, FJ697002	FJ697099
<i>M. nayaritense</i> Stuessy (4x)	1	M, Nayarit, 2008; JV, GF & EO, 1575.	FJ696992	FJ697091

Taxon (chromosome base number or ploidy level)	Accession	Collection details, voucher numbers	GenBank accession numbers	
			ITS	matK
<i>M. pringlei</i> B.L. Rob. (6x)	2	M, Nayarit, 2008; JV, GF & EO, 1577.	FJ696994–FJ696996	FJ697090
	3	M, Nayarit, 2008; JV, GF & EO, 1579.	FJ696993	FJ697092
	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18637.	FJ696990, FJ696991	FJ697097
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18650.	FJ696988	FJ697094
<i>M. sericeum</i> Lag. (6x)		M, Michoacán, 2005; TS, JV, CR & IC, 18572.	FJ696986, FJ696987	FJ697093
<i>M. strigosum</i> Stuessy (4x)	1	USA, Texas, Jeff Davis Co, 2005; CR & ML, 18728.	FJ696997, FJ696998	FJ697095
	2	M, Queretaro, 2006; TS, JV & CB, 19073.	FJ696999	FJ697096
<i>Ser. Cupulata</i>				
<i>M. appendiculatum</i> B.L. Rob. (2x)		M, Sonora, 2006; TS, JV & CB, 19046.	FJ697030	FJ697116
<i>M. cupulatum</i> A. Gray (2x)	1	M, Sinaloa, 2006; TS, JV & CB, 19044.	FJ697031	FJ697114
	2	M, Sonora, 2006; TS, JV & CB, 19048.	FJ697032	FJ697115
<i>M. glabribacteatum</i> Stuessy (2x)		M, Oaxaca, 2005; TS, JV, CR & IC, 18654.	FJ696989	FJ697100
<i>M. moctezumum</i> B.L. Turner		M, Sonora, 2006; TD & AR, 2007-706 (TEX).	FJ789805, FJ789806	FJ789803
<i>M. rosei</i> B.L. Rob. (2x)	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	–
<i>M. sinuatum</i> Brandegees (2x)		M, Baja California, 2006; TS & JV, 19037.	FJ697029	FJ697136
<i>M. tenellum</i> Hook.f. & Arn. (2x)	1	M, Nayarit, 2006; TS, JV, CB & EO, 19020.	FJ697028	FJ697117
	2	M, Nayarit, 2006; TS, JV, CB & EO, 19023.	FJ697027	FJ697118
<i>Ser. Longipila</i>				
<i>M. longipilum</i> B.L. Rob. (2x)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18630.	FJ696972, FJ696973	FJ697119
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18653.	FJ696974	FJ697120
<i>Sect. Bibractiaria</i> ($x = 14$)				
<i>M. bibracteatum</i> S. Watson (4x)	1	M, México, 2005; TS, JV, CR & IC, 18565.	FJ697056	FJ697145
	2	M, Durango, 2006; TS, JV, CR & CB, 19052.	FJ697057	FJ697146
<i>M. repens</i> Sessé & Moc. (2x, 4x)	1	M, Morelos, 2005; TS, JV, CR & IC, 18563.	FJ697059	FJ697147
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18639.	FJ697058	FJ697148
<i>Sect. Zarabellia</i> ($x = 9$)				
<i>M. gracile</i> Less. (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18586.	FJ697072	FJ697162
	2	M, Guerrero, 2005; TS, JV, CR & IC, 18674.	FJ697073	FJ697163
<i>M. longifolium</i> Cerv. ex Cav. (2x)	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
<i>M. microcephalum</i> Less. (2x)	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
<i>M. mimulifolium</i> B.L. Rob. (2x)		M, Oaxaca, 2005; TS, JV, CR & IC, 18656.	FJ697069	FJ697144
<i>M. paniculatum</i> Gardner (4x, 6x)	1	M, Chiapas, 2008; JV, EO & JC 1589.	FJ697065, FJ697066	–
	2	M, Chiapas, 2008; JV, EO & JC, 1591.	FJ697063	FJ697164

Taxon (chromosome base number or ploidy level)	Accession	Collection details, voucher numbers	GenBank accession numbers	
			ITS	matK
	3	M, Chiapas, 2008; JV, EO & JC, 1593.	FJ697064	FJ697165
	4	FL2935 (OS).	FJ697060–FJ697062	–
Sect. <i>Rhizomaria</i> ($x = 11$)				
<i>M. aureum</i> Brandegees (6x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18576.	FJ696970	FJ697151
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18635.	FJ696971	FJ697152
<i>M. montanum</i> Benth. var. <i>montanum</i> (2x)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18640.	FJ696967	FJ697153
<i>M. montanum</i> Benth. var. <i>viridulum</i> Stuessy (2x)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18646.	FJ696968	FJ697154
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18655.	FJ696969	FJ697155
Sect. <i>Alcina</i> ($x = 11$)				
<i>M. glabrum</i> S. Watson (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18598.	FJ697036	FJ697126
	2	M, Michoacán, 2005; TS, JV, CR & IC, 18624.	FJ697035	FJ697125
<i>M. nutans</i> Stuessy (2x)	1	M, Michoacán, 2005; TS, JV, CR & IC, 18591.	FJ697034	FJ697124
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18664.	FJ697033	FJ697123
<i>M. perfoliatum</i> Stuessy (Cav.) H.B.K. (2x)	1	M, Jalisco, 2005; TS, JV, CR & IC, 18604.	FJ697038	FJ697149
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18652.	FJ697037	FJ697150
Sect. <i>Serratura</i> ($x = 12$)				
<i>M. costaricense</i> Stuessy (4x)	1	CR, Prov. San José, 2006; TS, JV & IS, 19076.	FJ697051	FJ697129
	2	CR, Prov. San José, 2006; TS, JV & IS, 19084.	FJ697052	FJ697130
<i>M. dicoelocarpum</i> B.L. Rob. (2x, 4x)	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
<i>M. divaricatum</i> (Rich. in Pers.) DC. (2x)	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
	4	CR, Prov. San José, 2006; TS, JV & IS, 19086.	–	FJ697133
<i>M. northingtonii</i> B.L. Turner (4x)	1	M, Oaxaca, 2005; TS, JV, CR & IC, 18659.	FJ697054, FJ697055	FJ697139
	2	M, Oaxaca, 2005; TS, JV, CR & IC, 18660.	FJ697053	FJ697140
<i>M. sinaloense</i> Stuessy (4x)	1	M, Sinaloa, 2006; TS, JV, CB & EO, 19026.	FJ697048	FJ697127
	2	M, Sinaloa, 2006; TS, JV, CB & EO, 19027.	FJ697049, FJ697050	FJ697128
<i>M. tepicense</i> B.L. Rob. (2x)	1	M, Nayarit, 2005; TS, JV, CR & IC, 18615.	FJ697047	FJ697137
	2	M, Nayarit, 2005; TS, JV, CR & IC, 18617.	FJ697046	FJ697138
<i>Acanthospermum</i> ($x = 11$)				
<i>A. australe</i> Kuntze		Rauscher, 2002.	AF465844	–
<i>A. hispidum</i> DC.	1	A, Jujuy, 1993; TS & JM, 12956 WU.	FJ696965	FJ789804
	2	USA, Florida; LA, 3481, KSC.	FJ696964	–
<i>A. microcarpum</i> B.L. Rob.		Rauscher, 2002	AF465845	–
<i>Lecocarpus</i> ($x = 11$)				
<i>L. lecocarpoides</i> (B.L. Rob. & Greenm.) Cronquist & Stuessy		E, Galápagos, Osborn, 2001; CSB & HA, Lam1, DK.	–	FJ697078
<i>L. pinnatifidus</i> Decne.		E, Galápagos, Floreana, 2001; CSB & HA, Lam6, DK.	–	FJ697075
<i>L. sp.</i>		E, Galápagos; HA, s.n., DK & WU.	FJ696966	–
Outgroups				

Taxon (chromosome base number or ploidy level)	Accession	Collection details, voucher numbers	GenBank accession numbers	
			ITS	<i>matK</i>
<i>Acmella oppositifolia</i> (Lam.) R.K. Jansen		M, México, 2006; TS, JV, CB & EO, 19005.	–	FJ697074
<i>Galinsoga parviflora</i> Cav.		M, México, 2006; TS, JV, CB & EO, 19004.	FJ696962	FJ697076
<i>Milleria quinqueflora</i> L.		M, Jalisco, 2006; TS, JV, CB & EO, 19016.	FJ696961	FJ697077
<i>Siegesbeckia flosculosa</i> L'Hér.		Rauscher, 2002.	AF465888	–
<i>Smallanthus maculatus</i> (Cav.) H.Rob.		M, Querétaro, 2006; TS, JV & CB, 19072.	FJ696963	FJ697079
<i>Trigonospermum melampodioides</i> DC.		Rauscher, 2002.	AF465906	–

Table 2

Primers used for amplification and sequencing of ITS and *matK* 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., in press.
matK700 fwd.	5'-CAA TCT TCT CAC TTA CGA TCA ACA TC-3'	Gruenstaeudl et al., in press.
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., in press.
matK350 fwd.	5'-ATC TTC CCT AGA AAG GAA AGG GG-3'	Gruenstaeudl et al., in press.
matK1200 rev.	5'-TAT CAG AAT CTG ATA AAT CGG CCC-3'	Gruenstaeudl et al., in press.
matK1000 fwd.	5'-CCC TTG ACT TTC TGG GTT ATC G-3'	Gruenstaeudl et al., in press.
matK1450 rev.	5'-GAA GAA ACT CTT GGA AAG GTC AAG G-3'	Gruenstaeudl et al., in press.
matK1300 fwd.	5'-CTT GTG CTA GAA CTT TAG CTC GTA AG-3'	Gruenstaeudl et al., in press.
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)

Table 3Sequence statistics for ITS and *matK*.

	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	68.00	52.50	32.70	28.80	32.80
Coding rRNA regions	163–312	15.40	52.70	–	–	–
<i>matK</i> – <i>trnH</i>	1831–1904	19.74	33.94	7.30	6.50	5.80
<i>matK</i> gene	1479–1530	20.63	32.34	10.80	7.00	8.80

Abbreviations: GC% = GC-content in percent; MSD, maximum sequence divergence; IG, ingroup taxa; Ac.–Le., *Acanthospermum* and *Lecocarpus*; OG, outgroup taxa.

Table 4

Marginal likelihoods and their Monte Carlo error as well as the test statistic $2 \times \ln \text{BF}$ for several taxonomic hypotheses, tested separately for each marker. The compared hypotheses (unconstrained vs. alternative) are arranged in rows. $2 \times \ln \text{BF}_{\text{unconstrained vs. alternative}} < -10$ is regarded as strong support against the alternative hypothesis.

	Unconstrained	Monophyletic genus <i>Melampodium</i>	Monophyletic sect. <i>Melampodium</i>	Monophyletic sect. <i>Zarabellia</i>	Monophyletic sect. <i>Alcina</i>
ITS					
Marginal likelihood	-8621.74 (± 0.40)	-8636.06 (± 0.38)	-8648.73 (± 0.37)	-8633.86 (± 0.39)	-8650.60 (± 0.37)
$2 \times \ln \text{BF}$	–	-28.64	-53.98	-24.24	-57.72
<i>matK</i>					
Marginal likelihood	-7834.09 (± 0.27)	-7834.67 (± 0.30)	–	–	-7892.39 (± 0.32)
$2 \times \ln \text{BF}$	–	-1.16	–	–	-116.60