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# The Evolutionary History Of The White-Rayed Species Of *Melampodium* (Asteraceae) Involved Multiple Cycles Of Hybridization And Polyploidization[1]

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## Abstract

**Premise of the study—**Polyploidy plays an important role in race differentiation and eventually speciation. Underlying mechanisms include chromosomal and genomic changes facilitating reproductive isolation and/or stabilization of hybrids. A prerequisite for studying these processes is a sound knowledge on the origin of polyploids. A well-suited group for studying polyploid evolution consists of the three species of *Melampodium* ser. *Leucantha* (Asteraceae): *M. argophyllum*, *M. cinereum*, and *M. leucanthum*.

**Methods—**The origin of polyploids was inferred using network and tree-based phylogenetic analyses of several plastid and nuclear DNA sequences and of fingerprint data (AFLP). Genome evolution was assessed via genome size measurements, karyotype analysis, and *in situ* hybridization of ribosomal DNA.

**Key results—**Tetraploid cytotypes of the phylogenetically distinct *M. cinereum* and *M. leucanthum* had, compared to the diploid cytotypes, doubled genome sizes and no evidence of gross chromosomal rearrangements. Hexaploid *M. argophyllum* constituted a separate lineage with limited intermixing with the other species, except in analyses from nuclear ITS. Its genome size was lower than expected if *M. cinereum* and/or *M. leucanthum* were involved in its origin, and no chromosomal rearrangements were evident.

**Conclusions—**Polyploids in *M. cinereum* and *M. leucanthum* are of recent autopolyploid origin in line with the lack of significant genomic changes. Hexaploid *M. argophyllum* also appears to be of autopolyploid origin against the previous hypothesis of an allopolyploid origin involving the

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other two species, but some gene flow with the other species in early phases of differentiation cannot be excluded.

**Keywords**

allopolyploidy; Asteraceae; autopolyploidy; genome size; hybridization; *Melampodium*; polyploid evolution; rDNA



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Polyploidy is one of the major processes shaping angiosperm evolution (Wendel, 2000; Hegarty and Hiscock, 2007, 2008; Leitch and Leitch, 2008; Hufton and Panopoulou, 2009). This is evident from early whole-genome duplications in the ancestor of essentially all extant angiosperms (Wendel, 2000; Wolfe, 2001; Adams and Wendel, 2005a, b; De Bodt et al., 2005; Jiao et al., 2011) as well as from more recent poly ploidization events affecting most angiosperms (Masterson, 1994; Leitch and Bennett, 1997; Leitch and Leitch, 2008). Whereas autopolyploids originate from multiplication of the usually identical chromosome sets within a single (sub)species, allopolyploids derive from hybridization and multiplication of the usually differentiated chromosome sets coming from two or more progenitor (sub)species (Comai, 2005; Chen et al., 2007; Otto, 2007). Although the distinction between auto- and allopolyploids is difficult in polyploids exhibiting intermediate chromosome pairing behavior (expected polysomic inheritance in autopolyploids vs. disomic inheritance in allopolyploids; Ramsey and Schemske, 2002; Otto, 2007), both types are unambiguously known to occur frequently in nature (Tate et al., 2005; Soltis et al., 2007), where they often originate recurrently (Soltis et al., 2007; Dixon et al., 2009).

Polyploidy plays an important role in race differentiation and eventually speciation. Polyploidization leads to an instantaneous increase in the amount of genetic material upon which evolution can work, potentially leading to the evolution of new functions in duplicated genes (neofunctionalization; Ohno, 1970; Wolfe, 2001; Liu and Wendel, 2002). Furthermore, polyploids often show accelerated genomic evolution expressed as a higher rate of chromosome structural rearrangements and associated pairing behavior in meiosis (Weiss and Maluszyńska, 2000; Ramsey and Schemske, 2002; Otto, 2007; Schubert and Lysak, 2011), or genome size changes (Bennetzen and Kellogg, 1997; Bennetzen et al., 2005; Leitch and Leitch, 2008; Hawkins et al., 2008), contributing to the isolation of a polyploid from its lower-ploid ancestor(s). Finally, polyploidization contributes to stabilization of genomes after hybridization, a phenomenon common in plants (Anderson and Stebbins, 1954; Grant, 1981; Mallet, 2005, 2007; Rieseberg and Willis, 2007), by restoring regular meiotic pairing and fertility, thus creating crossing barriers with the parental taxa (allopolyploid speciation: Tate et al., 2005; Hegarty and Hiscock, 2007, 2008; Paun et al., 2009).

A prerequisite for studying processes associated with the evolution of polyploid genomes is a sound knowledge of their origin. Molecular sequence data have proven particularly useful for identifying the parental lineages of allopolyploids. Although incongruence between gene trees obtained from plastid DNA (cpDNA; inherited maternally in Asteraceae; Corriveau and Coleman, 1988; Harris and Ingram, 1991), and nuclear DNA (inherited from both parents; Sang et al., 1997; Hughes et al., 2002; Kim et al., 2008) provides evidence for

allopolyploidy, this approach is hampered by the often low variation in cpDNA sequences and by concerted evolution of the commonly used nuclear ribosomal internal transcribed spacer (nrITS) region (álvarez and Wendel, 2003). In low-copy nuclear genes, analyses of individual gene copies stemming from the maternal and paternal lineages can be recovered, rendering these genes a powerful and successful tool for inferring the origin of allo polyploids (Ferguson and Sang, 2001; Sang, 2002; Small et al., 2004; Lihová et al., 2006; Fortune et al., 2007; Kim et al., 2008; Shimizu-Inatsugi et al., 2009; Weiss-Schneeweiss et al., 2012).

A well-suited group for studying polyploid evolution is the white-rayed complex of *Melampodium* sect *Melampodium* ser. *Leucantha* (Asteraceae; Stuessy, 1972). Characterized by white instead of yellow or orange ray florets, it is a phylogenetically distinct group (Blöch et al., 2009) comprising three xerophytic subshrub species (*M. argophyllum*, *M. cinereum*, *M. leucanthum*) distributed in the arid regions of northern Mexico and the southwestern United States (Fig. 1; Stuessy, 1972; Stuessy et al., 2004). Like the entire sect. *Melampodium*, species of ser. *Leucantha* have a basic chromosome number of $x = 10$ (Weiss-Schneeweiss et al., 2009). The widespread *M. cinereum* and *M. leucanthum* comprise both diploid and tetraploid cytotypes (Fig. 1). These originated recurrently via autopolyploidy, as suggested by chromosomal studies and genetic data (Turner and King, 1964; Stuessy et al., 2004; Rebernig et al., 2010a, b). The third species of the complex is the hexaploid *M. argophyllum* (Stuessy, 1972; Stuessy et al., 2004), restricted to northeastern Mexico (Fig. 1). It shares morphological features with the two other species and has thus been hypothesized to be of allopolyploid origin involving *M. cinereum* and *M. leucanthum* (Stuessy et al., 2004). Thus, ser. *Leucantha* provides an excellent system to compare the type and extent of genomic changes in closely related polyploids with contrasting evolutionary histories.

Here, we used molecular sequence (plastid and nuclear DNA, including a low-copy gene), fingerprint (AFLPs), and restriction data in conjunction with karyological, and genome size data to investigate the origin and evolution of ser. *Leucantha* with special emphasis on *M. argophyllum*. Specifically, we wanted to (1) infer phylogenetic relationships among *M. argophyllum*, *M. cinereum*, and *M. leucanthum*, in particular to test the hypothesis of an allopolyploid origin for *M. argophyllum*; and (2) compare karyotype structure, genome size, and rDNA loci number and localization in polyploids of presumably different ages and modes of origin (recent auto- vs. ancient allopolyploidy).

## Materials And Methods

### Study species

*Melampodium argophyllum* occurs in western Nuevo León and adjacent Coahuila (Mexico) above 1830 m elevation (Stuessy, 1972; Fig. 1), where it is altitudinally separated from the otherwise sympatric *M. cinereum* var. *hirtellum* (Stuessy, 1971b). It flowers from February to October (Stuessy, 1972). *Melampodium argophyllum* shares morphological characters with both *M. cinereum* (similar leaf shape and head size) and *M. leucanthum* (similar outer phyllaries; Stuessy, 1971b) and has therefore been described as a variety of *M. cinereum* (Gray, 1884) as well as of *M. leucanthum* (Stuessy, 1971b), but is now again considered a

distinct species (Stuessy, 1972, following Blake, 1924). The species is known only from the hexaploid level ($2n = 6\times = 60$; Stuessy et al., 2004; Weiss-Schneeweiss et al., 2009).

*Melampodium cinereum* grows in mesquite shrublands in southern Texas and adjacent Mexico (Fig. 1). It differs from *M. leucanthum* and *M. argophyllum* by the outer phyllaries being connate only to one fourth their length (Stuessy, 1972). Based on morphological variation of the indumentum of leaves and stems, three varieties, *cinereum*, *hirtellum*, and *ramosissimum*, have been distinguished (Stuessy, 1971b, 1972), but genetic data suggest that var. *hirtellum* comprises two geographically separated entities (Rebernig et al., 2010b). Flowering time ranges from January to July, and despite some differences between varieties their flowering times overlap for at least 5 months. *Melampodium cinereum* comprises morphologically weakly and only quantitatively (Stuessy, 1972) distinguishable diploid and tetraploid cytotypes (with occasional tri-, penta-, or hexaploids; Stuessy et al., 2004; R. Obermayer et al., unpublished data), most prominently in var. *cinereum* (Stuessy et al., 2004; Rebernig et al., 2010b). The tetraploids occur exclusively in southeastern Texas largely to the exclusion of diploids, with no apparent ecological differentiation (Stuessy et al., 2004; Rebernig et al., 2010b).

*Melampodium leucanthum* grows on calcareous soils in arid habitats from Arizona and New Mexico to central Texas reaching the Edwards Plateau, extending northward to Colorado and Kansas and southward to northern Mexico (Stuessy, 1972; Fig. 1). It can be morphologically distinguished from the other two species by the outer phyllaries being united one half to three fourths their length (Stuessy, 1972). Despite evidence for three separate phylogeographic lineages (Rebernig et al., 2010a), no infraspecific morphological variants of taxonomic value are known. *Melampodium leucanthum* comprises morphologically indistinguishable diploid and tetraploid cytotypes (Turner and King, 1964; Stuessy, 1971a, b, 1972; Stuessy et al., 2004), with occasional tri, penta-, or hexaploids (Stuessy et al., 2004; Rebernig et al., 2010a). The tetraploids occupy a compact distribution area in eastern Texas around Austin to the (almost complete) exclusion of diploids (Stuessy et al., 2004).

## Plant material

Plant material was collected from 22 populations of *M. cinereum*, 90 populations of *M. leucanthum*, and two populations of *M. argophyllum* covering the whole distribution area of the three species (Rebernig et al., 2010a, b; Table 1). AFLP data have been generated for a large data set including all accessions of the white-rayed complex. Plastid and nuclear sequence data comprise a second smaller data set including a selected number of accessions of the species of ser. *Leucantha* (Table 1).

Achenes were collected from individual plants, where available, and seeds were germinated on wet filter paper for chromosome and genome size analyses. Plant leaf material collected in the field was dried and stored in silica gel until DNA isolation. Herbarium vouchers are deposited in the herbarium of the University of Vienna (WU) with duplicates in MEXU (Tables 1, 2; Appendix 1).

## Laboratory methods

Since most populations of *M. cinereum* and *M. leucanthum* form genetically cohesive groups (Rebernig et al., 2010a, b), we used one randomly selected individual per population of these two species for the final AFLP analyses. Additionally, eight individuals of *M. argophyllum* from the two available populations (Table 1) were added to previously published AFLP data sets (Rebernig et al., 2010a, b). Total genomic DNA was extracted, and AFLPs were generated and scored as described in Rebernig et al. (2010a, b), employing five primer combinations (fluorescent dyes in parentheses): *Eco* RI-ACA/*Mse* I-CAT (FAM), *Eco* RI-ACG/*Mse* I-CAA (VIC), *Eco* RI-ACC/*Mse*I-CAG (NED), *Eco* RI-ACT/*Mse*I-CAC (FAM), *Eco* RI-AGG/*Mse* I-CAA (VIC).

Sequences for three noncoding plastid regions (*psbA-trnH*, *rpl32-trnL*, *ndhF-rpl32*) were generated as described in Rebernig et al. (2010a, b). All sequences are deposited in GenBank (accession numbers in Table 1).

Amplification, cloning, and sequencing of the nuclear ITS regions followed the protocols described in Blöch et al. (2009). The complete 5S rDNA region including the nontranscribed spacer was amplified as described in Stuessy et al. (2011). The region between exons 11 and 16 of the low-copy nuclear gene *PgiC* (two paralogues in *Melampodium*; Stuessy et al., 2011) was amplified using newly developed specific primers (Stuessy et al., 2011).

The PCR products of the 5S rDNA spacer and the partial *PgiC* gene paralogues were cloned into pGEM-T-easy vector and transformed into JM109 competent cells (Promega, Vienna, Austria) according to the manufacturer's instructions. Inserts of 6–18 positive clones for the 5S rDNA spacer region (on average six clones per diploid genome) and 10–20 clones per diploid genome for *PgiC* 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, California, USA). The cycle sequencing reactions were performed using M13 universal primers, either in one or in both directions. Sequencing reactions were run on a 3130×l Genetic Analyzer automated sequencer (Applied Biosystems).

## Data analyses

AFLP data were analyzed using the neighbor-net method implemented in the program SplitsTree 4.8 (Huson and Bryant, 2006) based on Nei-Li distances (Nei and Li, 1979) calculated with the program FAMD 1.108 (Schlüter and Harris, 2006). Bootstrap analysis based on 2000 replicates was conducted in the program TREECON 1.3 (van de Peer and de Wachter, 1994).

Sequences were assembled using the program SeqMan II (DNAstar, Madison, USA), aligned manually or with the program Muscle 3.6 (Edgar, 2004) using default settings and further improved by visual inspection using the program BioEdit 7.0.9.0 (Hall, 1999). Prior

to all analyses of the cpDNA data, inversions in the plastid sequence data detected by visual inspection (*trnH-psbA* and *ndhF-rpl32*) were reverted (Rebernig et al., 2010a, b). In the 5S and the *PgiCII* data sets, clearly unalignable regions (combinations of mononucleotide repeats and microsatellites) in the 5′-end of the 5S rDNA spacer region (11–27 nucleotides) and the 3′-end of the *PgiCII* sequences (16–32 nucleotides) were excluded from further analyses. Likewise, a single recombinant *PgiCII* sequence was removed as well. Each data set was analyzed separately without outgroups, because the phylogenetic distance between ser. *Leucantha* and its presumptive sister group is relatively large and the introduction of distant lineages might have negative effects on phylogenetic inference (especially due to long-branch attraction) within ser. *Leucantha*. Maximum likelihood analyses were conducted using the program RAxML 7.2.2 (Stamatakis, 2006) employing the rapid bootstrap analysis plus maximum likelihood tree search (option f-a) with 1000 bootstrap replicates and a GTR+τ model (option-m GTRGAMMA). As phylogenetic trees are not suited for displaying reticulate patterns, the data were also analyzed using parsimony split networks (Bandelt and Dress, 1993) in the program SplitsTree 4.8 (Huson and Bryant, 2006) employing 1000 bootstrap replicates to assess support for splits. For this analysis, indels longer than 1 bp were recoded to be counted as single mutational steps only, and these indels were subsequently treated as a fifth character state.

### Amplification and restriction digest of ITS regions

The ITS sequence analyses supported the hypothesis of a hybrid origin of *M. argophyllum* (see Results). All three species of the white-rayed complex, therefore, including multiple individuals of *M. cinereum* (also those sharing plastid haplotypes with *M. leucanthum*; Rebernig et al., 2010b), were investigated in more detail via restriction patterns of ITS. Restriction patterns in already available ITS sequences (Blöch et al., 2009) were identified using Sequence Manipulation Suite (Stothard, 2000). The primers used for amplification were designed based on the ITS region sequence data set from Blöch et al. (2009) and were anchored in 18S and 26S rDNA for the entire ITS and in 5.8S and 26S rDNA for ITS2, respectively. These regions were amplified using 1× Reddy Mix PCR Master Mix (including 2.5 mmol/L $MgCl_2$; ABgene, Epsom, UK), 0.5 μmol/L each of forward and reverse primer, 0.8 μg/μL dimethyl sulfoxide (DMSO) and ca. 50 ng of DNA template in a T-CY thermocycler (Creacon Technologies, Emmen, Netherlands) under the following conditions: 3 min at 90°C; 35 cycles of 30 s at 94°C, 30 s at 52°C, and 45 s at 72°C; followed by 10 min at 72°C. The entire ITS region was digested with *Bst* UI (*Bsh* 12361; MBI-Fermentas) at 37°C for 16 h according to the manufacturer's protocol. The ITS2 region was digested with the FastDigest *Hae* III restriction enzyme (MBI-Fermentas) at 37°C for 10 min, following the manufacturer's recommendation. Resulting DNA fragments were separated electrophoretically (60 min at 80 V) on a 2% agarose gel.

### Ploidal level and genome size estimation

Measurements of DNA ploidal levels of all individuals used in the molecular analyses were conducted as described in Rebernig et al. (2010a, b). Additionally, absolute genome size was measured for three populations of diploid *M. cinereum* var. *hirtellum*, seven populations of *M. cinereum* var. *cinereum* (three diploid and four tetraploid populations), and 14 populations of *M. leucanthum* (eight diploid and six tetraploid populations), totaling 24

populations (Table 2). Approximately 10 mm $^2$ leaf tissue of *Glycine max* 'Idefix' (1C = 1.28 pg, Dolezel et al., 1998) and two seedlings of *Melampodium* were chopped together in 500 µL cold Otto I isolation buffer (Otto et al., 1981). After another 500 µL Otto I buffer was added, the nuclei solution was filtered through a 30 µL nylon mesh. Then 50 µL of RNase A (3 mg/mL; MBI-Fermentas) was added to each sample, which was incubated at 37°C for 30 min to remove RNA. The samples were incubated for ca. 90 min in Otto II buffer (Otto et al., 1981) containing propidium iodide (0.1 mmol/L; Sigma, Vienna, Austria). Analysis was conducted with a Partec CyFlow ML (Partec, Münster, Germany) equipped with a green laser beam, and each sample was measured four times to check for value accuracy. Due to insufficient number of viable achenes of *M. argophyllum*, its genome size was calculated from the relative fluorescence intensity values obtained from measurements of silica-gel dried leaf material (Rebernig et al., 2010a, b) using the 24 samples of *M. cinereum* and *M. leucanthum* with known genome sizes as calibration points (linear regression through the origin).

### Cytogenetic analyses

Chromosome numbers, karyotypes, and ideograms were established for selected accessions of all cytotypes and species of the white-rayed complex (Weiss-Schneeweiss et al., 2009). Fluorescent *in situ* hybridization (FISH) using 35S rDNA and 5S rDNA as probes followed protocols of Weiss-Schneeweiss et al. (2006, 2008). In *M. argophyllum*, FISH could not be performed due to the insufficient number of viable achenes.

## Results

### AFLP and sequence analysis

The five AFLP primer combinations chosen for the analysis generated 715 unambiguously scorable fragments in the size range from 100 to 500 bp: 167 with *Eco* RI-ACA/*Mse* I-CAT (FAM), 135 with *Eco* RI-ACG/*Mse* I-CAA (VIC), 123 with *Eco* RI-ACC/*Mse* I-CAG (NED), 160 with *Eco* RI-ACT/*Mse* I-CAC (FAM), and 130 with *Eco* RI-AGG/*Mse* I-CAA (VIC). All 113 individuals analyzed had a unique AFLP profile. The error rate (Bonin et al., 2004), based on comparisons among replicated individuals, amounted to 4%. The neighbor-net analysis of the AFLP data revealed a network of three well-supported (bootstrap support [BS] 100%) monophyletic groups corresponding to the three species (Fig. 2). The major split separated *M. leucanthum* from *M. cinereum* and *M. argophyllum*.

Relationships among the three species of ser. *Leucantha* were complicated and not always concordant among sequenced regions (sequence characteristics in Table 3). In all sequenced regions, tetraploid accessions of *M. cinereum* and *M. leucanthum* grouped with conspecific diploid accessions. Plastid sequence data revealed three well-supported groups (Fig. 3A, B). With the exception of some individuals of *M. cinereum* sharing haplotypes with *M. leucanthum* (this pattern is insensitive to sample size: data not shown; Rebernig et al., 2010b), these groups correspond to the three species.

Analyses of nuclear ITS sequences (Fig. 3C, D) inferred two groups (BS 94–98) corresponding to *M. cinereum* and *M. leucanthum*, each additionally containing sequences

of *M. argophyllum*. None of the three species was found to be monophyletic from analyses of 5S rDNA spacer sequences (Fig. 3E, F). Instead, the majority of clones isolated from *M. cinereum* and *M. leucanthum* fell together with some clones of *M. argophyllum* in a group (supported only in the maximum likelihood analysis), whereas the phylogenetic position of the remaining sequences from *M. cinereum* and *M. leucanthum* is unclear due to homoplasy (reticulations in the parsimony splits network: Fig. 3E) or poor resolution and insufficient support (maximum likelihood: Fig. 3F). The remaining sequences from *M. argophyllum* were distributed into two moderately to well-supported groups (Fig. 3E, F).

Relationships inferred from *PgiC* sequences suggested three groups with different degrees of cohesiveness and support (Fig. 3G-J). These largely corresponded to the three species with the exception of a few clones of *M. leucanthum* grouping with *M. cinereum* in *PgiCI* (Fig. 3G, H) and a single distinct clone of *M. cinereum* grouping with *M. argophyllum* in *PgiCII* (Fig. 3I, J). In both paralogues, sequences isolated from *M. argophyllum* fell into three distinct groups (Fig. 3G-J), although in *PgiCII* these were not found in all individuals studied. In *M. leucanthum*, lineage heterogeneity was found only in *PgiCI* (Fig. 3G, H).

## Restriction endonuclease digestion of ITS and ITS2

PCR amplifications of both the entire ITS region and of ITS2 alone always resulted in single bands of ca. 700 bp and ca. 350 bp, respectively (Fig. 4). Digestion of the entire ITS PCR product was always complete (Fig. 4A). In both cytotypes of *M. cinereum* var. *cinereum* (lanes 5–7), in eastern diploid var. *hirtellum* (lane 3; Rebernig et al., 2010b), and in diploid var. *ramosissimum* (lane 4) the digestion resulted in several bands, two of which were specific for *M. cinereum* (ca. 300 bp and ca. 200 bp). The other bands (ca. 500 bp, 150 bp and 100 bp; not all present in each population) were shared with *M. leucanthum*, where they are the only ones found (lanes 8–11). All analyzed individuals of western *M. cinereum* var. *hirtellum* (Rebernig et al., 2010b) had the same digestion pattern as *M. leucanthum* irrespective of whether they possessed a *cinereum* or a *leucanthum* cpDNA haplotype (lane 2, data not shown). All seven tetraploid individuals of *M. cinereum* var. *cinereum*, on the other hand, revealed the digestion pattern typical for *M. cinereum* irre spec tive of their cpDNA haplotype (lanes 6 and 7 and data not shown). All 10 individuals of *M. argophyllum* analyzed had almost identical banding patterns (lanes 12–15 and data not shown), sharing all bands specific for both *M. cinereum* and *M. leucanthum* .

The ITS2 region (ca. 350 bp) of *M. cinereum* (Fig. 4B; lanes 2–6) remained essentially undigested, whereas that of *M. leucanthum* was completely digested resulting in a single band due to the overlap of the two restriction products each of ca. 175 bp in length (lanes 7–11). Contrary to the results of the whole ITS digestion, the individuals of western *M. cinereum* var. *hirtellum* sharing haplotypes with *M. leucanthum* did not share the ITS2 restriction pattern with *M. leucanthum* (Fig. 4B). In most accessions of *M. argophyllum*, undigested and digested bands were observed, albeit often of different strength, a few individuals possessing only the *M. leucanthum*-specific band (lanes 11–15).

### Ploidal level and genome size estimation

In contrast to *M. cinereum* and *M. leucanthum*, which comprise two main ploidal levels each (DNA-diploids and DNA-tetraploids: Rebernig et al., 2010a, b), *M. argophyllum* was uniformly hexaploid (36 individuals; mean value given in Table 2). Flow cytometry measurements of genome size in newly germinated seedlings from selected populations showed no significant differences between varieties of *M. cinereum* ($t = 0.559$, $P = 0.61$; Table 2). Whereas diploid *M. cinereum* had a significantly smaller genome size than diploid *M. leucanthum* (2.22 pg/1C vs. 2.32 pg/1C, respectively; $t = -0.103$, $P < 0.001$), these differences disappeared at the tetraploid level (4.48 pg/1C vs. 4.52 pg/1C, respectively; $t = -0.034$, $P = 0.66$; Table 2). Monoploid genome sizes (C× values: Greilhuber et al., 2005) were not (*M. cinereum*) or only marginally significantly different (*M. leucanthum*) between cytotypes ($t = -0.028$, $P = 0.21$ and $t = 0.058$, $P = 0.05$, respectively). Due to the lack of fresh material, the genome size of *M. argophyllum* had to be calculated from the relative fluorescence values obtained from silica-gel dried leaf material using the regression Eq. 1C = 1.2785 × relative fluorescence ($R^2 = 1$). The thus-obtained mean genome size value for *M. argophyllum* (5.72 pg/1C; 1.9 pg/1C×; Table 2) is 14–16% lower than the expected additive value from an allopolyploid origin (span of 1C = 6.66 to 1C = 6.80 pg, depending on the assumed combination of parental diploid and tetraploid genomes) and 14–18% lower than the expected value from an autopolyploid origin of either species (based on mean genome sizes of current diploids).

### Cytogenetic analyses

*Melampodium cinereum* and *M. leucanthum* encompassed both diploid ($2n = 2× = 20$) and tetraploid ($2n = 4× = 40$) cytotypes (Fig. 5A-D). In tetraploids, aneuploid variation was observed in both species ($2n = 41$ and 42; Fig. 5C, D). As the size of the extra chromosomes in these aneuploid individuals was similar to that of the smallest chromosomes of the regular A-complement, it is not possible to determine whether the aneuploid numbers can be attributed to the presence of B-chromosomes or accessory A-chromosomes. *Melampodium argophyllum* was exclusively and regularly hexaploid ($2n = 6× = 60$; Fig. 5E).

The karyotypes of the diploid cytotypes of *M. cinereum* and *M. leucanthum* were very similar in morphology and structure, and nearly all chromosome pairs were distinguishable based on chromosome structure alone (Fig. 5A, B). Likewise, the tetraploid karyotypes of both species were very similar to one another and to those of the diploid cytotypes and did not show any apparent chromosomal rearrangements (Fig. 5A-D). However, sometimes the four homologues of each chromosome were differentiated into two pairs, rather than forming a group of four identical chromosomes, particularly in *M. cinereum* (Fig. 5C).

Molecular cytogenetic localization of 5S rDNA and 35S rDNA using FISH in diploid cytotypes revealed the presence of one locus of 35S rDNA and three loci of 5S rDNA (Fig. 5F-I). The single 35S rDNA locus (NOR) was found at a subterminal position in the short arm of chromosome 8. Two of the 5S rDNA loci were localized in the short arm of chromosome 5, a larger one in the subtelomeric region (locus 1) and a weaker one in the pericentromeric region (locus 2), while the third weak 5S rDNA locus (locus 3) was found in a pericentromeric position in the smallest submetacentric chromosome 10 (Fig. 5F-I).

Tetraploid cytotypes of both *M. cinereum* and *M. leucanthum* possessed twice as many loci with the same localization as the diploids (Fig. 5J, K). Although the 35S rDNA locus was present in all four chromosomes, it showed differentiation into two larger and two smaller signals, suggesting ongoing differentiation of the two homologous chromosome pairs. All four signals mark active loci, which in the interphase are decondensed and attached to the nucleolus/nucleoli (data not shown). The major 5S rDNA locus (locus 1) was clearly identifiable in all tetraploid individuals analyzed. The minor loci were also mostly present, but occasionally with one or two signals not clearly discernable in chromosomal spreads, particularly of locus 2 due to its close proximity to large locus 1 (Fig. 5J, K). As depending on the degree of chromosome condensation loci 1 and 2 were sometimes indistinguishable also in diploid plants (data not shown), there is no evidence for substantial rDNA loci loss in tetraploid genomes.

## Discussion

### Phylogenetic relationships and origin of M. argophyllum

*Melampodium cinereum* and *M. leucanthum* constitute well-separated and distinct gene pools (Fig. 2), in agreement with their morphological, geographical, and ecological separation (Stuessy, 1971b). Although currently no hybrids between the two species are known, their past evolution appears to have been shaped by multiple hybridization events and gene flow. Specifically, western *M. cinereum* var. *hirtellum* has experienced introgression from *M. leucanthum* as indicated by *leucanthum*-type plastid haplotypes and ITS sequences in at least some of the var. *hirtellum* populations (Figs. 3, 4). Whereas conversion of the 35S rDNA cistron is biased toward the maternal parent, even in the presence of extensive back-crossing (Lim et al., 2000), in western *M. cinereum* var. *hirtellum* ITS at least partially homogenized toward *M. leucanthum* irrespective of its parentage, a pattern observed also in other allopolyploid *Melampodium* species (Weiss-Schneeweiss et al., 2012). Repeated introgression of *M. leucanthum* into western var. *hirtellum* may explain its genetic distinctness from the morphologically indistinguishable eastern var. *hirtellum* (Rebernig et al., 2010b), but evidently this gene flow did not blur the border between *M. cinereum* and *M. leucanthum* (in line with a genic view of speciation; Wu, 2001). A second zone of introgression includes a few tetraploid *M. cinereum* var. *cinereum* populations in the east of the species' distribution, where distinct *leucanthum*-type haplotypes are present (Fig. 3A, B; Rebernig et al., 2010b). In these populations, the 35S rDNA cistron has not been affected, which may be related to an altogether lower amount of gene flow and strong backcrossing, or biased and parent-specific ribotype conversion (Arnold et al., 1988; Lim et al., 2008).

The situation for *M. argophyllum* is less clear. Plastid and, to a lesser extent, 5S rDNA and *PgiC* sequence data suggest *M. argophyllum* as a distinct third lineage. This agrees with a hypothesis put forward by Stuessy (1971b) based on morphological analyses that *M. argophyllum* resembles the progenitor of the whole complex. This renders *M. argophyllum* an altitudinally and ecologically isolated relict taxon of putative autopolyploid origin, possibly having arisen from the ancestral taxon of the whole complex. If so, the presence of three distinct groups in 5S rDNA and *PgiC* sequences might reflect independent evolution of

the three paraloguous loci that resulted from autopolyploidization likely involving diploid and autotetraploid parental cytotypes. Furthermore, sequences grouping with those from *M. cinereum* (Fig. 3) were either remnants of ancient polymorphisms or the results of introgression into *M. argophyllum*. Such introgressions may have occurred prior to polyploidization, because there is no evidence for current hybridization between *M. argophyllum* and any of the other two taxa. At present, this hexaploid species grows in a higher elevation rocky habitat and does not co-occur with *M. cinereum* or *M. leucanthum*, but there is still much to learn about *M. argophyllum*. It is confined to isolated mountains in northern Mexico that are difficult to access; therefore, the species has not yet been extensively sampled for cytological or genetic variation among existing populations.

Alternatively, *M. argophyllum* has been hypothesized to have originated via allopolyploidy from crosses between *M. cinereum* and *M. leucanthum* (Stuessy et al., 2004). A certain morphological intermediacy is reflected in previous classifications, which treated *M. argophyllum* as a variety either under *M. cinereum* (Gray, 1884) or *M. leucanthum* (Stuessy, 1969). Evidence for this hypothesis comes from nrITS sequence data (Figs. 3C, 3D, 4). As outlined already, *M. cinereum* and *M. leucanthum* apparently did hybridize repeatedly in their history. In general, formation and establishment of viable and fertile hybrids between different species on the diploid level is rather rare (Stebbins, 1958; Baack and Rieseberg, 2007; Mallet, 2005, 2007) due to genomic instability or hybrid sterility (Hegarty et al., 2005, 2006). Polyploidization following early hybridization may have resulted in genome stabilization (Hegarty and Hiscock, 2008) and restoration of fertility (Rieseberg, 2001; Salmon et al., 2005), resulting in allopolyploid speciation as known in several other cases in sect. *Melampodium* (Stuessy et al., 2011; Weiss-Schneeweiss et al., 2012).

Despite the use of several molecular markers, therefore, the mode of origin of *M. argophyllum* remains uncertain. Paradoxically, the sequence marker, nuclear ITS, considered most likely to lose signal for reticulation due to homogenization (álvarez and Wendel, 2003; Poczai and Hyvönen, 2009) is the only one showing strong evidence for an allopolyploid origin, whereas signal from the other markers is consistent with an autopolyploid origin with some gene flow. Evidence for an autopolyploid origin is also evident with the two *PgiC* paralogues, although their interpretation is complicated by intraindividual variation (due to, for instance, allelic variation, intraspecific gene duplications, or simply cloning artifacts; Brysting et al., 2011) and by partial failure to recover the expected number of copies in some individuals. Possibly the origin of *M. argophyllum* predates (full) separation of *M. cinereum* and *M. leucanthum*, when gene flow between paternal and descendant lineages still did occur. Not mutually exclusive, the origin of *M. argophyllum* may involve both auto- and allopolyploidization, but further data will be necessary to test these hypotheses.

## Genome evolution

Members of ser. *Leucantha* have significantly larger genomes than those of the presumptive sister group ser. *Cupulata* (ca. 2.2-fold increase; Weiss-Schneeweiss et al., 2012) or any other species group within section *Melampodium* (H. Weiss-Schneeweiss et al., unpublished data). This may be connected to different life histories of this group (perennial and

xerophilous life form) because annuals tend to have lower nuclear DNA content than perennials (Bennett, 1972). Previous studies have found positive correlations between the duration of the vegetative period and genome sizes in various angiosperms (Bennett, 1987; Turpeinen et al., 1999). Additionally, the shift toward arid environments in ser. *Leucantha* may have triggered additional genome size increase (Knight and Ackerly, 2002; Knight et al., 2005), possibly via transposable element mobility induced by environmental stress (Fontdevila, 2005; Vitte and Panaud, 2005). Although correlations of water stress and genome size are usually negative, in some plant groups they have been shown to be positive (Knight and Ackerly, 2002).

Autopolyploidization in *M. cinereum* and *M. leucanthum* did not significantly affect genome size, which is within the range of the expected additive values (Table 2). This pattern agrees with that seen in young autopolyploids of other plant groups (e.g., Ozkan et al., 2006; Eilam et al., 2009). In contrast to nascent autopolyploids, established autopolyploids (i.e., those which usually are cytologically already diploidized) were often reported to show substantial genome reorganization compared with their diploid relatives (Eilam et al., 2010; Parisod et al., 2010). In contrast to the young autopolyploids of *M. cinereum* and *M. leucanthum*, *M. argophyllum* has a lower genome size than would be expected irrespective of the mode of origin (autopolyploidy vs. allopolyploidy). This is congruent with the likely older age of this polyploid and thus agrees with the general trend of genome downsizing in polyploids (Leitch and Bennett, 2004; Weiss-Schneeweiss et al., 2006; Lysak et al., 2009). Alternatively, it may reflect smaller genome size of its ancestor(s), which remain unknown at this point, as is conceivable given the evidence for genome size increase in the evolution of the entire ser. *Leucantha* from its relatives.

The karyotype in ser. *Leucantha*, as assessed by chromosome morphology as well as by number and localization of 5S and 35S ribosomal RNA genes (no data available for *M. argophyllum*), is very stable both across species and across ploidal levels. This supports the relatively recent origin of polyploids in *M. cinereum* and *M. leucanthum* as suggested previously (Stuessy et al., 2004, Rebernig et al., 2010a, b). Only in tetraploid *M. cinereum*, and to a lesser extent in hexaploid *M. argophyllum*, some of the homologous chromosomes can form distinguishable pairs/groups (Fig. 5) as seen in other groups (e.g., *Spartina*, Aïnouche et al., 2004; *Gossypium*, Adams and Wendel, 2004). This suggests ongoing diploidization of the tetraploids, likely acting as one of the processes that restore fertility and allow further evolution of the genomes (Eilam et al., 2009).

## APPENDIX 1

Sampling location coordinates, voucher (collector) number, number of analyzed individuals (*N*), and ploidal level of populations of species of the white rayed complex analyzed with AFLPs.

| Voucher | Location | *N* | Ploidal level |
|---|---|---|---|
| *Melampodium leucanthum* | | | |
| 18676 | N 30.194 W 30.1938 | 1 | 2× |

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| Voucher | Location | *N* | Ploidal level |
|---|---|---|---|
| 18677 | N 30.216 W 98.238 | 1 | 2× |
| 18679 | N 30.227 W 98.382 | 1 | 2× |
| 18681 | N 30.280 W 98.906 | 1 | 2× |
| 18682 | N 30.170 W 98.382 | 1 | 2× |
| 18683 | N 29.891 W 98.407 | 1 | 4×* |
| 18686 | N 29.616 W 98.756 | 1 | 4× |
| 18687 | N 29.529 W 98.848 | 1 | 4× |
| 18709 | N 30.616 W 97.860 | 1 | 4× |
| 18710 | N 30.694 W 97.981 | 1 | 4× |
| 18711 | N 30.696 W 98.253 | 1 | 4× |
| 18712 | N 30.671 W 98.255 | 1 | 4× |
| 18714 | N 30.387 W 98.366 | 1 | 4× |
| 18720 | N 29.718 W 101.364 | 1 | 4×* |
| 18725 | N 30.789 W 104.033 | 1 | 2× |
| 18726 | N 31.005 W 104.825 | 1 | 2× |
| 18727 | N 30.999 W 103.755 | 1 | 2×* |
| 18729 | N 31.352 W 103.579 | 1 | 2× |
| 18730 | N 31.640 W 102.634 | 1 | 2× |
| 18731 | N 31.698 W 102.572 | 1 | 2× |
| 18732 | N 31.934 W 101.865 | 1 | 2× |
| 18733 | N 31.871 W 101.646 | 1 | 2× |
| 18734 | N 31.900 W 100.716 | 1 | 2× |
| 18735 | N 32.964 W 102.011 | 1 | 2× |
| 18737 | N 32.288 W 102.611 | 1 | 2× |
| 18738 | N 31.852 W 103.114 | 1 | 2× |
| 18753 | N 35.326 W 102.369 | 1 | 2× |
| 18756 | N 35.003 W 101.918 | 1 | 2× |
| 18757 | N 34.986 W 101.717 | 1 | 2× |
| 18758 | N 36.221 W 101.333 | 1 | 2× |
| 18760 | N 36.449 W 100.372 | 1 | 2× |
| 18761 | N 36.427 W 99.883 | 1 | 2× |
| 18762 | N 35.845 W 100.396 | 1 | 2× |
| 18764 | N 35.432 W 100.770 | 1 | 2× |
| 18768 | N 33.860 W 100.851 | 1 | 2× |
| 18769 | N 34.219 W 100.888 | 1 | 2× |
| 18770 | N 34.380 W 101.110 | 1 | 2× |
| 18771 | N 34.788 W 100.898 | 1 | 2× |
| 18772 | N 35.009 W 100.895 | 1 | 2× |
| 18774 | N 31.226 W 99.757 | 1 | 2× |
| 18776 | N 31.781 W 99.779 | 1 | 4× |
| 18778 | N 31.761 W 98.899 | 1 | 4× |
| 18779 | N 31.689 W 98.813 | 1 | 4× |

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| Voucher | Location | *N* | Ploidal level |
|---|---|---|---|
| 18781 | N 31.358 W 98.129 | 1 | 4× |
| 18782 | N 31.170 W 98.183 | 1 | 4× |
| 18783 | N 30.928 W 98.001 | 1 | 4× |
| 18785 | N 31.064 W 97.571 | 1 | 4× |
| 18786 | N 31.575 W 97.834 | 1 | 4× |
| 18787 | N 31.793 W 98.227 | 1 | 4× |
| 18800 | N 33.238 W 110.253 | 1 | 2× |
| 18801 | N 34.002 W 111.313 | 1 | 2× |
| 18802 | N 33.480 W 111.443 | 1 | 2× |
| 18805 | N 33.962 W 111.863 | 1 | 2× |
| 18807 | N 34.349 W 112.153 | 1 | 2× |
| 18808 | N 34.605 W 111.857 | 1 | 2× |
| 18809 | N 34.617 W 111.843 | 1 | 2× |
| 18810 | N 34.650 W 111.760 | 1 | 2× |
| 18811 | N 34.639 W 111.807 | 1 | 2× |
| 18812 | N 34.712 W 111.880 | 1 | 2× |
| 18813 | N 34.730 W 111.968 | 1 | 2× |
| 18814 | N 34.755 W 112.091 | 1 | 2× |
| 18816 | N 24.747 W 112.105 | 1 | 2× |
| 18817 | N 34.825 W 111.778 | 1 | 2× |
| 18819 | N 34.756 W 111.765 | 1 | 2× |
| 18820 | N 35.121 W 113.666 | 1 | 2× |
| 18822 | N 32.602 W 110.744 | 1 | 2× |
| 19056 | N 28.228 W 101.056 | 1 | 2×* |
| 20000 | N 32.263 W 107.232 | 1 | 2× |
| 20002 | N 31.953 W 107.675 | 1 | 2× |
| 20003 | N 32.640 W 107.954 | 1 | 2× |
| 20005 | N 32.786 W 108.139 | 1 | 2× |
| 20007 | N 32.953 W 107.489 | 1 | 2× |
| 20010 | N 33.275 W 107.281 | 1 | 2× |
| 20011 | N 34.146 W 106.907 | 1 | 2× |
| 20014 | N 33.793 W 106.274 | 1 | 2× |
| 20016 | N 34.009 W 105.941 | 1 | 2× |
| 20017 | N 34.945 W 106.191 | 1 | 2× |
| 20021 | N 35.288 W 106.216 | 1 | 2× |
| 20029 | N 35.230 W 103.767 | 1 | 2× |
| 20030 | N 35.395 W 104.179 | 1 | 2× |
| 20031 | N 34.897 W 104.718 | 1 | 2× |
| 20032 | N 34.036 W 104.747 | 1 | 2× |
| 20033 | N 32.490 W 104.348 | 1 | 2× |
| 20034 | N 32.529 W 103.801 | 1 | 2× |
| 20035 | N 32.506 W 103.126 | 1 | 2× |

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| Voucher | Location | *N* | Ploidal level |
|---|---|---|---|
| 20038 | N 30.239 W 103.380 | 1 | 2× |
| 20039 | N 29.784 W 103.177 | 1 | 2× |
| 20040 | N 29.516 W 103.403 | 1 | 2× |
| 20043 | N 31.003 W 104.824 | 1 | 2× |
| 20044 | N 31.610 W 104.857 | 1 | 2× |
| 20045 | N 31.315 W 106.078 | 1 | 2× |
| 20046 | N 32.397 W 106.614 | 1 | 2× |
| *M. argophyllum* | | | |
| 19059 | N 26.249 W 100.895 | 6 | 6×* |
| 19060 | N 26.232 W 100.677 | 2 | 6×* |
| *M. cinereum var. cinereum* | | | |
| 18689 | N 28.608 W 99.841 | 1 | 2×* |
| 18690 | N 28.035 W 99.529 | 1 | 2× |
| 18691 | N 27.781 W 99.451 | 1 | 2× |
| 18693 | N 26.890 W 99.262 | 1 | 2× |
| 18694 | N 26.697 W 99.109 | 1 | 4× |
| 18697 | N 26.823 W 98.855 | 1 | 4× |
| 18698 | N 26.941 W 98.619 | 1 | 4× |
| 18699 | N 27.195 W 98.622 | 1 | 4× |
| 18700 | N 27.264 W 98.486 | 1 | 4×* |
| 18701 | N 27.278 W 98.734 | 1 | 4× |
| 18702 | N 27.232 W 98.934 | 1 | 4× |
| 18704 | N 27.405 W 98.576 | 1 | 4× |
| 18705 | N 27.513 W 98.481 | 1 | 4× |
| 18706 | N 27.733 W 98.263 | 1 | 4× |
| 18707 | N 27.870 W 98.088 | 1 | 4× |
| 18708 | N 28.242 W 98.106 | 1 | 4× |
| *M. cinereum var. hirtellum* | | | |
| 19054 | N 27.945 W 101.257 | 1 | 2× |
| 19055 | N 27.929 W 101.343 | 1 | 2× |
| 19057 | N 28.193 W 101.072 | 1 | 2× |
| 19058 | N 26.509 W 101.352 | 1 | 2× |
| 19061 | N 26.366 W 100.122 | 1 | 2× |
| 19062 | N 26.375 W 100.025 | 1 | 2×* |
| *M. cinereum var. ramosissimum* | | | |
| 19063 | N 25.181 W 98.199 | 1 | 2×* |
| 19065 | N 24.816 W 98.185 | 1 | 2× |

* *Notes*: Populations counted chromosomally (other determined only by flow cytometry).

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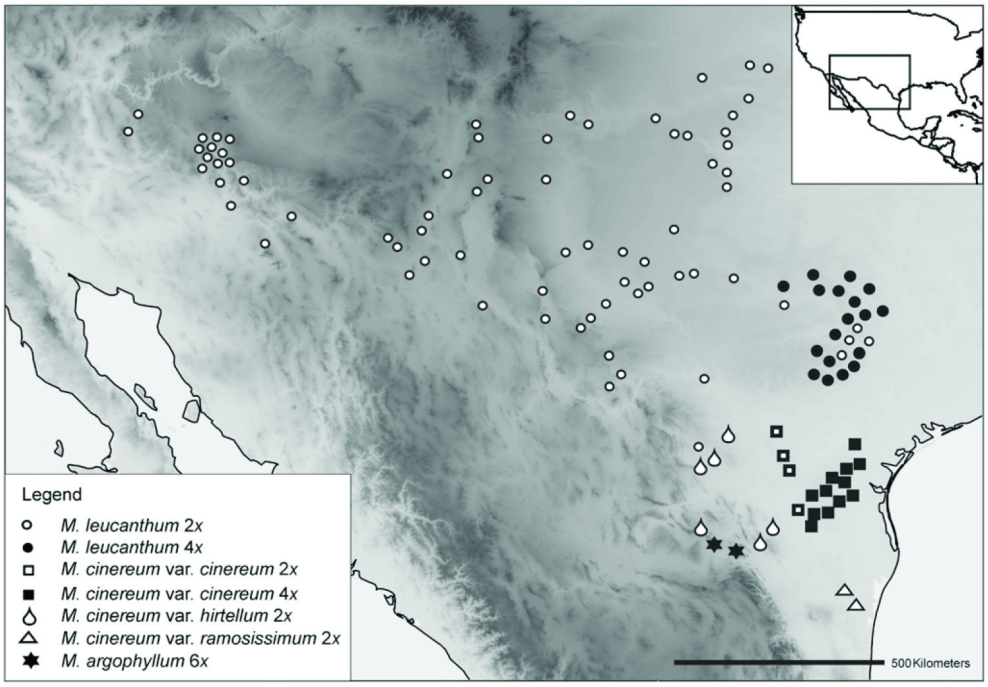


**Fig. 1. Distribution map of the analyzed populations of the white-rayed complex of *Melampodium.***

The collection area represents the entire distribution of the complex.




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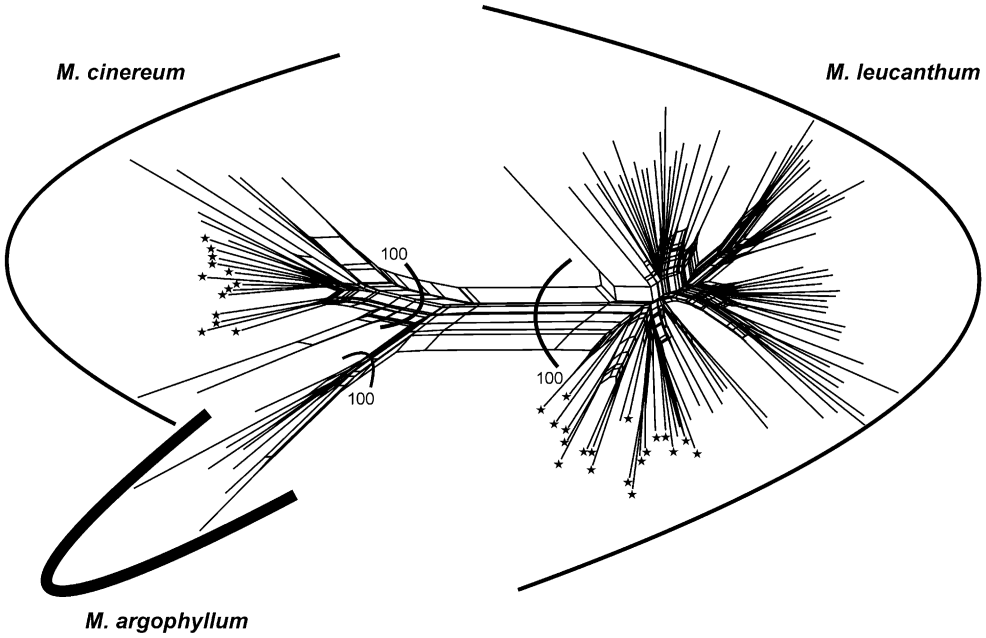


**Fig. 2. Neighbor-net for the AFLP data using Nei-Li distances.**
Numbers indicate bootstrap values from a neighbor joining analysis. Asterisks indicate tetraploid cytotypes.


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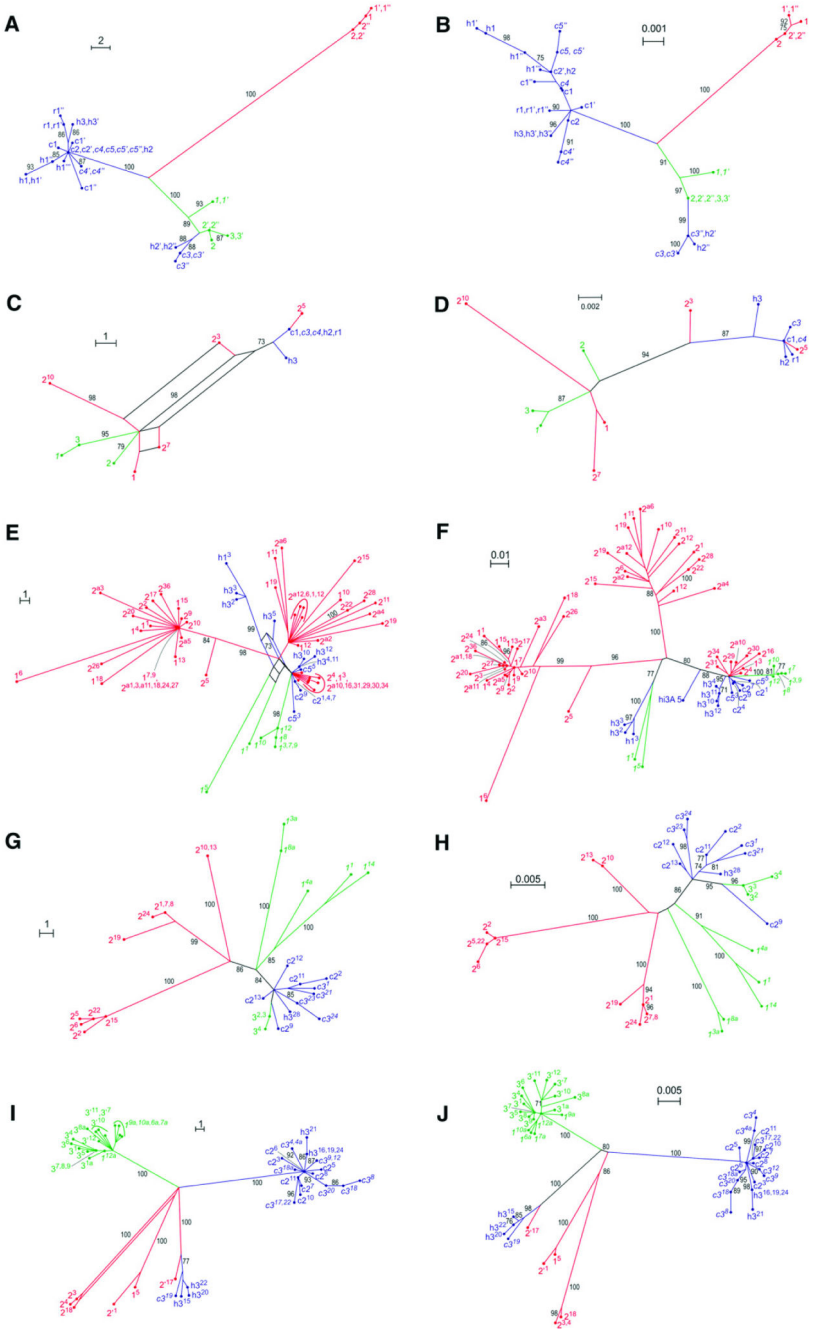


**Fig. 3. Genealogical relationships of nuclear and plastid DNA sequences of *Melampodium cinereum*(blue; the three varieties *cinereum, hirtellum*, and *ramosissimum* indicated by their initials), *M. leucanthum* (green) and *M. argophyllum* (red) inferred via parsimony splits networks (A, C, E, G, I) and maximum likelihood trees (B, D, F, H, J).**

(A, B) Plastid data, (C, D) nuclear ITS, (E, F) 5S rDNA nontranscribed spacer, (G, H) *PgiC* copy I, (I, J) *PgiC* copy II. Numbers at splits or branches are bootstrap support values of 70 or higher. Population numbers as in Table 1, different individuals from the same population indicated by apostrophes. In (E-J), different clones are designated with superscript numbers.




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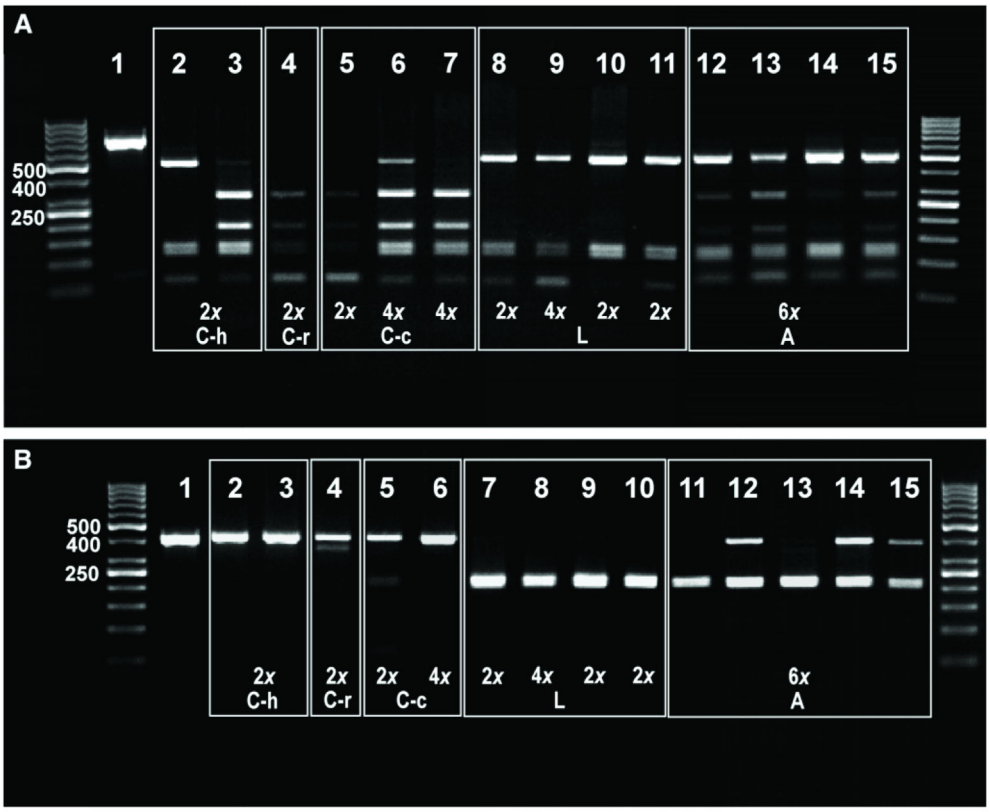


**Fig. 4. Restriction patterns for ITS from *Melampodium*.**

(A) The entire ITS digested with *Bst* UI: 1, undigested ITS amplification product; 2, *M. cinereum* var. *hirtellum* 19057C, 2×; 3, *M. cinereum* var. *hirtellum* 19061B, 2×; 4, *M. cinereum* var. *ramosissimum* 19065H, 2×; 5, *M. cinereum* var. *cinereum* 18688H, 2×; 6, *M. cinereum* var. *cinereum* 18697F, 4×; 7, *M. cinereum* var. *cinereum* 18694J, 4×; 8, *M. leucanthum* 18814B, 2×; 9, *M. leucanthum* 18785G, 4×; 10, *M. leucanthum* 20033C, 2×; 11, *M. leucanthum* 19056B, 2×; 12, *M. argophyllum* 19059E, 6×; 13, *M. argophyllum* 19060A, 6×; 14, *M. argophyllum* 19060CI, 6×; 15, *M. argophyllum* 19060E, 6×. (B) ITS2 only digested with *Hae* III: 1, undigested ITS2 amplification product; 2, *M. cinereum* var. *hirtellum* 19057B, 2×; 3, *M. cinereum* var. *hirtellum* 19061B, 2×; 4, *M. cinereum* var. *ramosissimum* 19065H, 2×; 5, *M. cinereum* var. c *inereum* 18688H, 2×; 6, *M. cinereum* var. *cinereum* 18694N, 4×; 7, *M. leucanthum* 18814B, 2×; 8, *M. leucanthum* 18785G, 4×; 9, *M. leucanthum* 20033C, 2×; 10, *M. leucanthum* 19056B, 2×; 11, *M. argophyllum* 19059A, 6×; 12, *M. argophyllum* 19059E, 6×; 13, *M. argophyllum* 19059I, 6×; 14, *M. argophyllum* 19060A, 6×; 15, *M. argophyllum* 19060C, 6×. Abbreviations: C-h, *M. cinereum* var. *hirtellum*; C-r, *M. cinereum* var. *ramosissimum*; C-c, *M. cinereum* var. *cinereum*; L, *M. leucanthum*; A, *M. argophyllum*. Unlabelled lanes contain markers (DNA ladder).

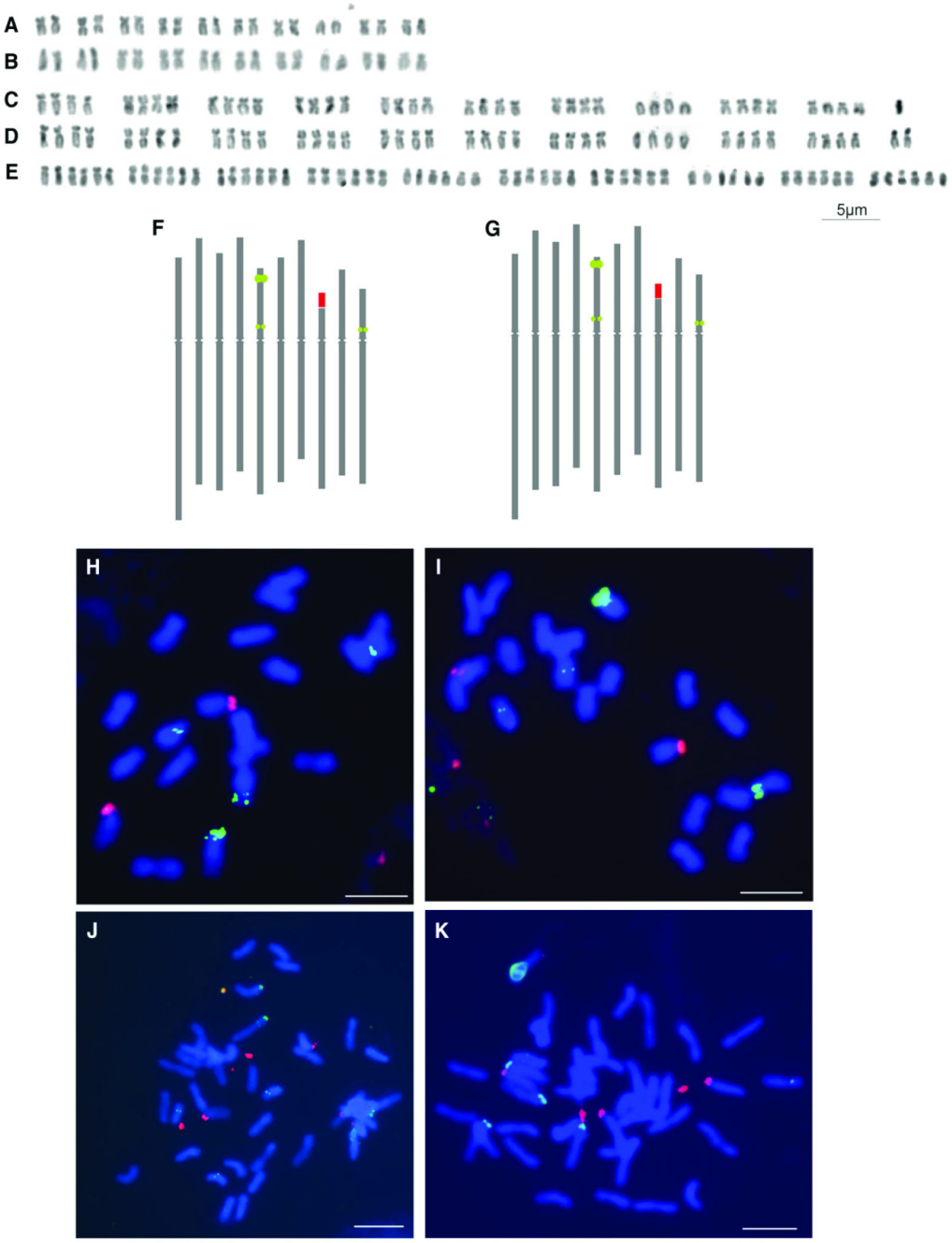


**Fig. 5. Chromosomal analyses of the white-rayed species of *Melampodium*.**

Karyotypes (A–E) and idiograms (F, G) of *M. cinereum* var. *cinereum* (A, F: 2×, *18688*; C: 4×, *18703*), *M. leucanthum* (B, G: 2×, *19056*; D: 4× *18683*), and *M. argophyllum* (E: 6×; *19059*). (H–K) *In situ* chromosomal localization of 5S rDNA (green signals) and 35S rDNA loci (red signals) in diploid (*18690*; H) and tetraploid (*18700*; J) *M. cinereum* var. *cinereum*, as well as diploid (*19056*; I) and tetraploid (*18683*; K) *M. leucanthum*. Scale bar (H–K) = 5 µm.




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**Table 1**

***Melampodium*** **species names, localities, voucher numbers, ploidal levels (taken from Weiss-Schneeweiss et al., 2009), and GenBank accession numbers of analyzed taxa.**

All vouchers deposited in WU and MEXU; Countries: M, Mexico; USA, United States of America. Collectors: CB, C. Blöch; CR, C. A. Rebernig; EO, E. Ortiz; JV, J. L. Villaseñor; MB, M. Barfuss; TS, T. F. Stuessy.

| Taxon | Accession number | Locality | Ploidal level | ITS | *psb*A-*trn*H, *ndh*F-*rpl*32, *rpl*32-*trn*L | 5S rDNA spacer | *PgiCI* | *PgiCII* |
|---|---|---|---|---|---|---|---|---|
| *M. argophyllum* (A.Gray ex B.L.Rob.) S.F.Blake (6×) | 1 | M, Nuevo León, 2006; TS, JV, EO & CB, 19059. | 6× | FJ697009 | FJ845873–FJ845875; FJ845867–FJ845869; FJ845879–FJ845881 | JF277454–JF277466 | – | JF277549 |
| | 2 | M, Nuevo León, 2006; TS, JV, EO & CB, 19060. | 6× | FJ697010–FJ697013 | FJ845876–FJ845878; FJ845870–FJ845872; FJ845882–FJ845884 | JF277419–JF277453 | JF277491–JF277502 | JF277544–JF277548 |
| *M. cinereum* DC. var. *cinereum* (2×, 4×) | 1 | USA, Texas, Frio Co, 2005; TS & CR, 18688. | 2× | FJ697006 | FJ456097–FJ456099; FJ456020–FJ456022; FJ456174–FJ456176 | – | – | – |
| | 2 | USA, Texas, Webb Co., 2005; TS & CR, 18690. | 2× | – | FJ456103–FJ456104; FJ456026–FJ456027; FJ456180–FJ456181 | JF277413–JF277416 | JF277503–JF277507 | JF277550–JF277556 |
| | 3 | USA, Texas, Zapata Co, 2005; TS & CR, 18694. | 4× | FJ697008 | FJ456112–FJ456114; FJ456035–FJ456037; FJ456189–FJ456191 | – | JF277508–JF277511 | JF277533–JF277543 |
| | 4 | USA, Texas, Jim Hogg Co, 2005; TS & CR, 18698. | 4× | FJ697007 | FJ456118–FJ456120; FJ456041–FJ456043; FJ456195–FJ456197 | – | – | – |
| | 5 | USA, Texas, Jim Hogg Co., 2005; TS & CR, 18701. | 4× | – | FJ456127–FJ456129; FJ456050–FJ456052; FJ456204–FJ456206 | JF277417–JF277418 | – | – |
| *M. cinereum* DC. var. *hirtellum* Stuessy (2×) | 1 | Mexico, Coahuila, 2006; TS, JV, EO & CB, 19054. | 2× | – | FJ456149–FJ456152; FJ456072–FJ456075; FJ456226–FJ456229 | JF277404 | – | – |
| | 2 | M, Coahuila, 2006; TS, JV, EO & CB, 19057. | 2× | FJ697015 | FJ456156–FJ456158; FJ456079–FJ456081; FJ456233–FJ456235 | – | – | – |
| | 3 | M, Nuevo León, 2006; TS, JV, EO & CB, 19061. | 2× | FJ697014 | FJ456162–FJ456164; FJ456085–FJ456087; FJ456239–FJ456241 | JF277397–JF277403 | JF277512 | F277557–JF277564 |
| *M. cinereum* DC. var. *ramosissimum* DC. (A.Gray) (2×) | 1 | M, Tamaulipas, 2006; TS, JV & CB, 19063. | 2× | FJ697016 | FJ456168–FJ456170; FJ456091–FJ456093; | – | – | – |
| | 2 | M, Tamaulipas, 2006; TS, JV & CB, 19064. FJ456245–FJ456247 | 2× | FJ697017 | – | – | – | – |

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| Taxon | Accession number | Locality | Ploidal level | ITS | *psb*A-*trn*H, *ndh*F-*rpl*32, *rpl*32-*trn*L | 5S rDNA spacer | *PgiCI* | *PgiCII* |
|---|---|---|---|---|---|---|---|---|
| *M. leucanthum* Torr. & A.Gray (2×, 4×) | 1 | USA, Texas, Medina Co, 2005; TS & CR, 18687. | 4× | FJ697005 | FJ846101, FJ846102; FJ845899, FJ845900; FJ846303, FJ846304 | JF277405–JF277412 | JF277483–JF277487 | F277515–JF277519 |
| | 2 | USA, Arizona, Graham Co, 2006; CR & MB, 18800. | 2× | FJ697004 | FJ846189–FJ846191; FJ846987–FJ846989; FJ846391–FJ846393 | – | – | – |
| | 3 | USA, Arizona, Yavapai Co, 2006; CR & MB, 18808. | 2× | FJ697003 | FJ846200, FJ846201; FJ846998, FJ846999; FJ846402, FJ846403 | – | JF277488–JF277490 | F277520–JF277532 |

## Table 2

**Genome size estimation (1C; pg) for selected populations of *Melampodium cinereum* and *M. leucanthum* (A) and genome size extrapolation for *M. argophyllum* (B).**

| Taxon | Pop. no. | Ploidal level | Genome size (1C; pg) | Genome size (1C×; pg) |
|---|---|---|---|---|
| A) *M. cinereum* and *M. leucanthum* | | | | |
| *M. cinereum* var. *hirtellum* | 19055 | 2× | 2.19 | 2.19 |
| *M. cinereum* var. *hirtellum* | 19058 | 2× | 2.26 | 2.26 |
| *M. cinereum* var. *hirtellum* | 19062 | 2× | 2.17 | 2.17 |
| Mean ± SD | | | | 2.21 ± 0.05 |
| *M. cinereum* var. *cinereum* | 18689 | 2× | 2.23 | 2.23 |
| *M. cinereum* var. *cinereum* | 18690 | 2× | 2.24 | 2.24 |
| *M. cinereum* var. *cinereum* | 18692 | 2× | 2.20 | 2.20 |
| Mean ± SD | | | | 2.22 ± 0.02 |
| *M. cinereum* var. *cinereum* | 18694 | 4× | 4.46 | 2.23 |
| *M. cinereum* var. *cinereum* | 18697 | 4× | 4.45 | 2.23 |
| *M. cinereum* var. *cinereum* | 18700 | 4× | 4.46 | 2.23 |
| *M. cinereum* var. *cinereum* | 18708 | 4× | 4.56 | 2.28 |
| Mean ± SD | | | | 2.24 ± 0.03 |
| *M. leucanthum* | WGS83 MLK | 2× | 2.28 | 2.28 |
| *M. leucanthum* | 18722 | 2× | 2.31 | 2.31 |
| *M. leucanthum* | 18727 | 2× | 2.32 | 2.32 |
| *M. leucanthum* | 18774 | 2× | 2.34 | 2.34 |
| *M. leucanthum* | 18800 | 2× | 2.35 | 2.35 |
| *M. leucanthum* | 18808 | 2× | 2.35 | 2.35 |
| *M. leucanthum* | 20000 | 2× | 2.28 | 2.28 |
| *M. leucanthum* | 20030 | 2× | 2.31 | 2.31 |
| Mean ± SD | | | | 2.32 ± 0.03 |
| *M. leucanthum* | 18683 | 4× | 4.53 | 2.27 |
| *M. leucanthum* | 18709 | 4× | 4.61 | 2.31 |
| *M. leucanthum* | 18712 | 4× | 4.57 | 2.28 |
| *M. leucanthum* | 18720 | 4× | 4.27 | 2.14 |
| *M. leucanthum* | 18779 | 4× | 4.66 | 2.33 |
| *M. leucanthum* | 18780 | 4× | 4.46 | 2.23 |
| Mean ± SD | | | | 2.26 ± 0.07 |

| Taxon | Pop. no. | Ploidal level | Rel. fluor. | 1C/1C× (pg) |
|---|---|---|---|---|
| B) *M. argophyllum* | | | | |
| *M. argophyllum* | 19059 | 6× | 4.4 ± 0.17 | 5.68/1.89 |
| *M. argophyllum* | 19060 | 6× | 4.4 ± 0.01 | 5.75/1.92 |
| Mean ± SD | | | | 5.72 ± 0.05/1.9 ± 0.02 |

*Notes*: Extrapolated values are given for 1C/1C×. Relative fluorescence (Rel. fluor.) values are from flow cytometry measurements of 35 individuals of silica-gel dried leaf material. Pop. no. = population number.

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**Table 3**

**Sequence characteristics of DNA regions studied in *Melampodium* ser. *Leucantha***

| Character | Plastid regions | 5S rDNA spacer | ITS | *PgiCI* | *PgiCII* |
|---|---|---|---|---|---|
| Alignment length unedited / edited | 2233 / 2233 | 538 / 499 | 791 / 791 | 702 / 702 | 741 / 679 |
| Number of variable characters | 65 | 283 | 34 | 111 | 153 |
| Number of parsimony-informative characters | 59 | 163 | 19 | 67 | 97 |
| Maximum likelihood (ln) | −3383.37 | −3950.33 | −1385.19 | −1782.19 | −2133.99 |