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HYBRIDIZATION, POLYPLOIDY, AND THE EVOLUTION OF SEXUAL SYSTEMS IN *MERCURIALIS* (EUPHORBIACEAE)

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Abstract.—Hybridization and polyploidy are widely believed to be important sources of evolutionary novelty in plant evolution. Both can lead to novel gene combinations and/or novel patterns of gene expression, which in turn provide the variation on which natural selection can act. Here, we use nuclear and plastid gene trees, in conjunction with morphological data and genome size measurements, to show that both processes have been important in shaping the evolution of the angiosperm genus *Mercurialis*, particularly a clade of annual lineages that shows exceptional variation in the sexual system. Our results indicate that hexaploid populations of *M. annua*, in which the rare sexual system androdioecy is common (the occurrence of males and hermaphrodites) is of allopolyploid origin involving hybridization between an autotetraploid lineage of *M. annua* and the related diploid species *M. huetii*. We discuss the possibility that androdioecy may have evolved as a result of hybridization between dioecious *M. huetii* and monoecious tetraploid *M. annua*, an event that brought together the genes for specialist males with those for hermaphrodites.

Key words.—Androdioecy, heterogeneous ITS, hybridization, morphology, phylogeny.

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Evolutionary transitions from one sexual system to another represent one of the most profound biological changes a lineage can undergo. Not only do they constitute a change in an important life-history trait with immediate implications for individual fitness, but they also affect the context, rate, and direction of the future evolution of all other traits (Barrett 2002). Transitions between combined (e.g., hermaphroditism) and separate (e.g., dioecy) sexes are particularly interesting, because they represent not only a shift in the way individuals allocate limited resources to one or both sexual functions, but also in the geometry of pollen and seed dispersal shadows and potentially in the rate of selfing versus outcrossing (Lloyd 1982; Charlesworth 1999).

The causes of evolutionary transitions between different sexual systems have attracted a great deal of theoretical and empirical research (Charlesworth 1999; Webb 1999). Various, not necessarily exclusive, selective mechanisms have been hypothesized, and these can be broadly summarized in terms of: (1) the benefits of inbreeding avoidance by unisexual individuals compared with the potential benefits enjoyed by self-compatible hermaphrodites (Lloyd 1975; Charlesworth and Charlesworth 1978; Thomson and Barrett 1981); (2) the benefits to males or females of sexual specialization, compared with the benefits of sharing resources between both sexual functions (Charnov et al. 1976; Givnish 1980; Bawa and Beach 1981; Niklas 1985; Seger and Eckhart 1996); and (3) the combined costs or benefits of both outcrossing and specialization (Charlesworth and Charlesworth 1981; Freeman et al. 1997; Charlesworth 1999; Barrett 2002). However, whatever its causes, a shift from one sexual system to another almost certainly requires a change in the ecological or genetic context of the population in question, from a scenario that favors one strategy to one favoring an alternative. From a genetic point of view, polyploidy and hybridization probably represent two of the more extreme ways in which genomic architecture, and thus the genetic context of selection, can be altered.

The radical change in the genomic structure of populations through polyploidization can have immediate consequences for levels of inbreeding depression, for the maintenance of any self-incompatibility mechanism, or for floral morphology; such factors can change conditions that favor one sexual system over another (Pannell et al. 2004). Thus, a change in floral morphology and/or the breakdown of self-incompatibility through polyploidization (Westergaard 1958; Stone 2002; but see Mable 2004) can cause a direct increase in the selfing rate, and, if inbreeding depression is lower in the polyploid genome, increased selfing can be subsequently selected (e.g., Charlesworth and Charlesworth 1978; Lande and Schemske 1985; Lande et al. 1994; Schultz 1999). By the same token, a shift in the mating system toward increased self-fertilization can improve the probability that a new polyploid lineage is able to establish (Levin 1975; Rausch and Morgan 2005), because selfing prevents ovules from being fertilized by incompatible pollen from the ancestral (diploid) population. We therefore expect evolution to be influenced by potentially complex interactions between polyploidy and the sexual system (Pannell et al. 2004).

From the perspective of transitions between hermaphroditism and dioecy, there is good comparative evidence, both among (Miller and Venable 2000, 2002) and within species (Yeung et al. 2005), that polyploidization can trigger the evolution of separate sexes. Thus, Miller and Venable (2000) found that polyploid dimorphic lineages have evolved from diploid hermaphroditic ancestors at least 20 times within at least 12 different genera of flowering plants, and the same association between polyploidy and sexual dimorphism has been documented amongst populations of the phylogenetic species *Lycium californicum* (Yeung et al. 2005). In these

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species, dioecy is thought to have evolved as an outcrossing mechanism following the loss of self-incompatibility upon polyploidization (Baker 1984; Miller and Venable 2000; Charlesworth 2001). In the genus *Empetrum*, the polyploid subspecies *E. nigrum* ssp. *hermaphroditum* is hermaphroditic, whereas its diploid relative *E. nigrum* ssp. *nigrum* is dioecious (cited in Richards 1997). An association between polyploidy and the sexual system was also evident in dioecious *Silene* and *Rumex*, in which artificially induced autopolyploids yielded hermaphrodite progeny (Westergaard 1958). Finally, polyploidy is associated with a transition from dioecy to hermaphroditism in the annual species of the European genus *Mercurialis* (Euphorbiaceae) (Durand 1963; Durand and Durand 1992; Pannell et al. 2004). In many of these situations, we might expect polyploid hermaphrodites to be able to establish more easily amongst diploid neighbors by self-fertilization (Levin 1975; Rausch and Morgan 2005).

Variation in sexual systems and polyploidy in annual *Mercurialis* species (annual mercuries) is interesting from several points of view. Not only do they provide an example of the evolution of functional hermaphroditism (monoecy) from dioecy with polyploidization, but they also present a rare instance of the co-occurrence of dioecy with an annual life history. The annual mercuries are also unusual in that they display not only dioecy and monoecy within the single *M. annua* species complex, but also because many populations of *M. annua* are androdioecious; that is, males coexist with hermaphrodites (Durand and Durand 1985, 1992; Pannell 1997a). Theoretical models predict that androdioecy should be much harder to evolve and maintain than its more common female analogue, gynodioecy (Charlesworth and Charlesworth 1978; Charlesworth 1984; Pannell 2002). The relatively recent discovery of androdioecy in several species, including *M. annua*, has thus prompted new hypotheses about how it might be maintained, and several empirical studies have been undertaken to test these hypotheses (reviewed in Pannell 2002).

Briefly, hypotheses for the maintenance of androdioecy invoke selection for reproductive assurance during colonization as a factor favoring self-compatible monoecy, selection for sexual specialization favoring dioecy under a regime of wind-pollination in large populations, and selection under an intermediate regime of recurrent colonization favoring androdioecy (Pannell 1997b, 2001). Similar ideas invoking the selective advantage of reproductive assurance have been hypothesized for the origin of monoecy from the breakdown of dioecy via androdioecy (Wolf and Takebayashi 2004). There is some evidence that differential rates of population turnover among metapopulations or regions explain the geographic variation in sexual systems of *M. annua* (Eppley and Pannell 2006; Obbard et al. 2006a). There is also phylogenetic or comparative support for the idea that androdioecy has evolved from dioecy rather than monoecy or hermaphroditism in several other unrelated species of animals or plants (reviewed in Pannell 2002). However, although substantial progress has been made in understanding the maintenance of different sexual systems in *M. annua*, we are still largely ignorant about the polarity of evolutionary transitions between combined versus separate sexes in the group, and particularly about the phylogenetic origin of androdioecy itself.

In *M. annua*, dioecy would appear to be the ancestral state, if only because dioecious populations are diploid and monoecious and androdioecious populations are polyploid (Durand and Durand 1992). However, it is unclear whether androdioecy evolved from dioecy, or as a reversion toward separate sexes from monoecy. The preponderance of dioecy in the genus *Mercurialis* suggests a dioecious origin for monoecy and androdioecy in *M. annua* (Pannell 2001), but phylogenetic analysis has not previously been extended to include samples from an androdioecious lineage (Krähenbühl et al. 2002). Moreover, our recent discovery of the dioecious tetraploid species *M. canariensis*, which appears on morphological grounds to be closely related to *M. annua*, indicates that the relation between the sexual system and polyploidy is more complex in *Mercurialis* than previously thought (Obbard et al. 2006b). Significantly, polyploidy and hybridization are common within the genus (Pax 1914; Krähenbühl et al. 2002), and *M. annua* itself is a polyploid complex (Durand 1963; Durand and Durand 1992). We were thus prompted to consider the role that polyploidization and hybridization may have played in the evolution of sexual systems in this genus (Pannell et al. 2004), because both these processes are widely recognized as potential sources of evolutionary novelty during plant evolution generally (Levin 1983, 2002; Rieseberg et al. 2003).

In this paper, we reconstruct nuclear and plastid gene trees, using ITS and chloroplast DNA sequences, respectively, to infer the nature and direction of sexual-system evolution within the monophyletic clade comprising the annual species of *Mercurialis*. In particular, we identify reticulate relationships within the clade using both intraindividual variation in ITS sequences (e.g., Popp and Oxelman 2001; Hughes et al. 2002; Rauscher et al. 2002; Devos et al. 2005; Neves et al. 2005), as well as differences in topology between nuclear and plastid gene trees (e.g., Palmer et al. 1983; Soltis et al. 1991, 1995). We complement our molecular phylogenetic reconstruction with genome-size and morphometric analysis, because both of these types of data can be informative in studies of hybridization and polyploidy (e.g., Rieseberg and Ellstrand 1993; Ohri 1998; Horandl and Greilhuber 2002; Levin 2002).

Materials and Methods

Study Species

All species of *Mercurialis* are wind pollinated, the majority are dioecious (Table 1), and all except *M. leiocarpa* are native to Europe and the Mediterranean Basin (Tutin et al. 1968; Krähenbühl et al. 2002). The genus is composed largely of perennial taxa, but there are several weedy annual lineages that we find form a single clade comprising the *M. annua* polyploid complex, diploid *M. huetii*, and the new tetraploid species *M. canariensis*. *Mercurialis huetii*, which is found in relatively undisturbed habitat and has a narrow distribution in northeastern Spain and southern France, is morphologically very similar to *M. annua*, but the two can be distinguished on the basis of architectural traits (Durand 1963; Obbard et al. 2006b). The *M. annua* complex comprises populations ranging from diploid to 12-ploid. In contrast with *M. huetii*, *M. annua* occupies highly disturbed, anthropogenic

TABLE 1. Species of the genus *Mercurialis*, with sexual-system and life-history attributes.

Species	Ploidy	Sexual system	Life history
M. annua L.	2× ($x = 8$)[1]	dioecious	annual
	4×–12× ($x = 8$)[1]	monoecious or androdioecious	annual
M. canariensis D. J. Obbard & S. A. Harris	4× ($x = 8$)	dioecious	annual
M. huetii Hanry.	2× ($x = 8$)[1]	dioecious	annual
M. perennis L.	6×–12× ($x = 8$)[2]	dioecious	rhizomatous perennial
M. ovata Sternb. & Hoppe.	2×–4× ($x = 8$)[2]	dioecious	rhizomatous perennial
M. leiocarpa Sieb. & Zucc.	2× ($x = 8$)[2]	monoecious	rhizomatous perennial
	6× ($x = 8$)[2]	(not reported, but monoecy and dioecy both known in the complex)	rhizomatous perennial
M. elliptica Lam.	$2n = 42$, $2n = 220$[3]	dioecious	woody perennial
M. corsica Cosson.	$2n = 66$[3]	dioecious	woody perennial
M. tomentosa L.	$2n = 26$[3]	dioecious	woody perennial
M. reverchonii Rouy.	$2n = 26$[3]	dioecious	woody perennial

[1] Durand 1963. [2] Krähenbühl and Küpfer 1995. [3] Krähenbühl et al. 2002.

ruderal habitats (Durand 1963). *Mercurialis canariensis* has a limited distribution in the Canary Islands; it is larger and more robust than *M. annua* and is easily distinguished by its large recurved stipules and the presence of bracts under the male flowers (Obbard et al. 2006b).

Durand (1963) recognized three different species within the *M. annua* complex: *M. annua* sensu stricto, comprising only dioecious diploid populations, *M. ambigua* L., comprising monoecious and androdioecious tetraploid and hexaploid populations, and *M. monoica* (Moris) Durand, comprising populations with higher ploidy that are invariably monoecious. However, this taxonomy seems arbitrary and, although diploids can generally be distinguished from polyploids on the basis of the presence or absence of monoecious plants, the different polyploid lineages are morphologically almost indistinguishable (Durand and Durand 1985; Obbard et al. 2006b). We believe that the annual species of *Mercurialis* would benefit from a rigorous modern taxonomic treatment. However, in the absence of a biologically helpful classification, we refer to all members of the complex as *M. annua* sensu lato (s.l.), noting polyploid level where necessary.

Sampling

Our sampling included diploid, tetraploid and hexaploid individuals of *M. annua*; individuals of *M. huetii* and *M. canariensis*; as well as individuals of the woody perennial species *M. elliptica*, *M. perennis*, *M. tomentosa*, and *M. reverchonii*. Samples for the annual species were selected to cover the largest possible geographic and morphological ranges (Fig. 1; Appendix available online only at http://dx.doi.org/10.1554/06-104.1.s1). For sequence-based phylogenetic analysis, genomic DNA was extracted from plants grown from seed collected at the following locations (Fig. 1; online Appendix): (1) diploid *M. annua* (populations 0002, 0059, 0061, 0080), (2) tetraploid *M. annua* (1018, 1020, 1031), (3) hexaploid *M. annua* (0011, 0020, 0058, 0060), (4) *M. canariensis* (0091, 0200, 0209), and (5) *M. huetii* (0678, 0719). Where the same population appears in our presentation of the results for both the ITS and cpDNA analyses, the same individual was used. We used silica-dried leaf material for DNA extractions from *M. elliptica*, *M. perennis*, and *M. tomentosa*, whereas *M. reverchonii* DNA was extracted from herbarium specimens (online Appendix). In the recent analysis of Wurdack et al. (2005), the genus *Mercurialis* is placed in a core acalyphoid clade, sister to a clade comprising *Lobanilia*, *Claoxylon*, *Micrococca*, and relatives. However, because no members of this sister clade were available to us, we followed Krähenbühl et al. (2002) in using *Ricinus communis* (also identified as a core acalyphoid; Wurdack et al. 2005) as an outgroup.

Our morphological analysis used 26 populations sampled in the following five groups (Fig. 1; online Appendix): (1) diploid *M. annua* (populations 1564, 0002, 0596, 0062, 0074, 0079, 0228, 0232); (2) tetraploid *M. annua* (populations 1018, 1020, 1031); (3) hexaploid *M. annua* (populations 0012, 0620, 0631, 0636, 0648, 0660, 0085, 1036, 1044); (4) *M. canariensis* (populations 0200, 0206, 0209, 0213); and (5) *M. huetii* (populations 0678, 0719). Although the individuals used were not those used for the molecular phylogenetic analysis, they were grown using seed from the same collections. With the exception of population 0232, genome size measurements were made on all the populations used in the morphometric analysis.

DNA Content

DNA content was measured by flow cytometry on three plants from each population. For each individual, approximately 15 mg of leaf material was used, along with 5 mg of leaf material from *Lycopersicon esculentum* (cv. Gardener's Delight) as an internal standard. Leaf material was chopped with a razor blade in 1 ml ice-cold LB01 lysis buffer and staining solution, modified from Dolezel et al. (1989): 15 mM Tris base, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, 15 mM β-mercaptoethanol, 50 μg ml^{-1} propidium iodide and 50 μg ml^{-1} RNAse. The resulting suspension was filtered through 30 μm mesh CellTric disposable filter (Partec GmbH, Münster, Germany) and analyzed with a Becton Dickinson (Franklin Lakes, NJ) FACScan flow cytometer. For each sample, 5000 events were recorded in each of five runs,

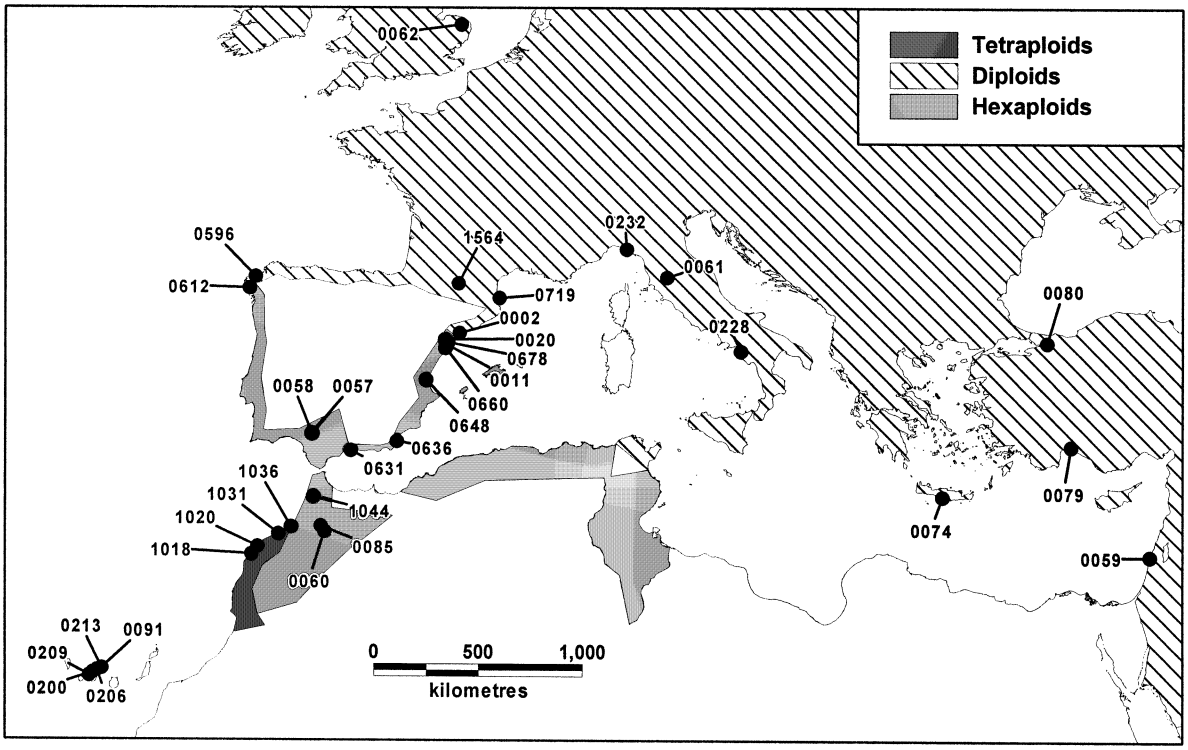

FIG. 1. Distribution of the annual species of *Mercurialis* in Europe, with the origin of samples used in this study. Hatched areas show the approximate distribution of *M. annua* diploids, tetraploids, and hexaploids; a complex polyploid swarm up to 12× occurs in Corsica and Sardinia (not shown). *Mercurialis huetii* grows in southeastern France and northwestern Spain. Numbers refer to populations used in the morphometric, DNA content, and phylogenetic studies. Map modified from Durand (1963).

and mean peak values were evaluated using CellQuest software (Becton Dickinson). The coefficient of variation for each peak used was above 2.00 and below 5.00. For each run, the mean DNA content of the sample peak was calculated with reference to *L. esculentum*, which has a DNA content of 4.10 pg (Bennett and Leitch 2003). Linearity of the flow cytometer was confirmed using a suspension of 2.0×10^7 chicken erythrocyte nuclei ml^{-1} fixed in ethanol-phosphate buffered saline (PBS; Biosure, Grass Valley, CA) and diluted 1:10 in a staining solution consisting of calcium- and magnesium-free Dulbecco's PBS, 0.05 mg ml^{-1} propidium iodide and 0.6% (v/v) Nonidet P40 (Sigma-Aldrich Corp., St. Louis, MO). All C-values have been submitted to the Royal Botanic Gardens (RBG; Kew, U.K.) C-value database.

Chromosome Counts

Where possible, population polyploid level was inferred from flow cytometry of DNA content, with cross checks by reference to the sexual system and geographic location (using the extensive survey of Durand 1963). When direct chromosome counts were used in addition to flow cytometry (e.g., for tetraploid and several hexaploid populations of *M. annua* and for *M. canariensis*), two to five mitotic cells were used. Seeds were germinated in the dark on moist filter paper at room temperature. Root tips, 1–3 cm long, were harvested and treated with 0.2% (w/v) colchicine for 3 h, then fixed in ethanol:acetic acid (3:1) for a minimum of 24 h. The fixed root tips were incubated at 60°C for 10 min in 1M HCl, stained with Schiff's reagent, and examined using standard procedures.

DNA Isolation, Polymerase Chain Reaction, and Sequencing

DNA was extracted from fresh and dried leaf material according to the modified CTAB procedure of Doyle and Doyle (1987). Leaves were ground under liquid nitrogen in microcentrifuge tubes, and, after incubation in 2× CTAB at 65°C, the samples were purified with two chloroform:isoamyl alcohol (24:1) extractions. Following propan-2-ol precipitation at −20°C, samples were washed in 76% ethanol, dried, and resuspended in water. Samples were stored at −20°C.

For all polymerase chain reactions (PCR), the reagents were as follows: 2.5 μM each of dATP, dTTP, dGTP, and dTCP, 0.8 μM of each primer, 1 U DNA polymerase and 10–100 ng DNA. Reaction volumes were 25 or 50 μl. All primer sequences and reaction conditions are shown in Table 2. Following amplification, PCR products were checked for homogeneity on an agarose gel and then purified using QIAquick purification spin columns (Qiagen Ltd, Crawley, U.K.) according to the manufacturer's instructions. Sequencing reactions were performed in both directions for each PCR product using BigDye version 3.1 (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. In all cases, the sequencing reactions used the same primers as the amplification PCRs. The sequencing products were analyzed using an ABI Prism DNA Sequencer 3730 (Applied Biosystems).

TABLE 2. Polymerase chain reaction (PCR) primers and conditions.

Region	Primer sequence	PCR conditions
*trn*L-*trn*F noncoding		35 cycles of 1 min at
e[1]	5′-GGT TCA AGT CCC CTC TAT CCC-3′	94°C, 1 min at 53°C
f[1]	5′-ATT TGA ACT GGT GAC ACG AG-3	and 2 min at 72°C
*mat*K-*trn*K 5′ intron		3 min at 95°C; 35 cycles
trnK−2R[2]	5′-CCC GGA ACT AGT CGG ATC-3′	of 1 min at 95°C, 1
1908F[2]	5′-GGC ATC CCA TTA GTA AGC-3′	min at 50°C and 1 min
		at 72°C; 7 min at 72°C
ITS ''universal''		40 cycles of 1 min at
ITSL[3]	5′-TCG TAA CAA GGT TTC CGT AGG TG-3′	97°C, 1 min at 48°C
ABI102[4]	5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′	and 2 min at 72°C; 7
		min at 72°C
ITS *M. huetii* specific (1a)		2 min at 97°C; 30 cycles
Huet60MMF[5]	5′-TCC GCG CCC CTC ATT CTC C**T**G **A**CG a**G**-3′	of 30 sec at 97°C then
ABI102i[5]	5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′	60 sec at 74°C; 3 min
		at 74°C
ITS *M. huetii* specific (1b)		2 min at 97°C; 30 cycles
ITSL[3]	5′-TCG TAA CAA GGT TTC CGT AGG TG-3′	of 30 sec at 97°C, 35
Huet468MMR[5]	5′-AAC ATA **A**AT TTT GGG CCA ACC ACA TGa **A**-3′	sec at 69°C and 35 sec
		at 72°C; 3 min at 72°C
ITS *M. canariensis* exclusion (2a)		2 min at 97°C; 30 cycles
Ann47MMF[5]	5′-**T**AG T**C**G GG**T** GAA TTT GTG GCT **CC**a C-3′	of 30 sec at 97°C then
ABI102i[5]	5′-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3′	60 sec at 74°C; 3 min
		at 74°C
ITS *M. canariensis* exclusion (2b)		2 min at 97°C; 30 cycles
ITSL[3]	5′-TCG TAA CAA GGT TTC CGT AGG TG-3′	of 30 sec at 97°C, 35
Ann459MMR[5]	5′-**C**MG ACG GCT **A**AG AAC AGC GCA CGt **C**-3′	sec at 66°C and 35 sec
		at 72°C; 3 min at 72°C

[1] Taberlet et al. (1991). [2] Lavin et al. (2000). [3] Hsiao et al. (1994). [4] L. Lledo (Royal Botanic Gardens, Kew). [5] Designed for present study, internal to ITS ''universal'' primers with the intention of excluding ITS sequences from specific taxa (see main text); bold bases are mismatches to the excluded template, and bases in lowercase are mismatches to all potential templates.

Identification of Intraindividual Variation in ITS

To identify additional divergent ITS sequences present in putative hybrids but not amplified by universal primers, we designed specific primers based on sequences obtained using universal primers (for a discussion of this approach see Rauscher et al. 2002). Following Cha et al. (1992), primers were designed for maximum stringency by placing mismatches with the excluded sequences at the 3′ end, and incorporating an additional mismatch in the penultimate 3′ base. Specific forward and reverse primers covering the 3′ end of ITS1, 5.8S RNA, and 5′ end of ITS2 were designed for two purposes: 1a and 1b to amplify *M. huetii* ITS DNA, but to exclude *M. annua* sequences amplified by the universal primers (Table 2); 2a and 2b to amplify diploid *M. annua* ITS DNA, but to exclude the *M. canariensis* tetraploid sequences amplified by the universal primers (Table 2). The specific primers were internal to the sequences amplified using universal primers, allowing them to be paired with universal primers and thus to provide two overlapping amplicons that covered the full length of the ITS1-ITS2 region.

DNA Sequence Analysis

The aligned sequences have been submitted to GenBank as population sets (for accession numbers, see online Appendix). Chloroplast sequences *trn*L-*trn*F and *mat*K-*trn*K were concatenated and analyzed separately from ITS-5.8S rDNA sequences. Both Bayesian (MrBayes ver. 3.1.1, Ronquist and Huelsenbeck 2003) and maximum parsimony (PAUP*, Swofford 2002) methods were used for phylogeny reconstruction. Parsimony analysis used a heuristic search with tree bisection and reconnection, treating gaps as both missing data and as characters (Simmons and Ochoterena 2000). Bayesian analysis was performed using both complex and simple models of sequence evolution. First, we assumed a general time-reversible nucleotide substitution model with gamma-distributed rate variation, using a single partition for the ITS dataset and five separate partitions for the cpDNA dataset (*trn*L-*trn*F spacer, *mat*K-*trn*K intron, *mat*K codon positions one to three). Second, we applied a single parameter substitution model (i.e., Jukes-Cantor) to a single partition for each of the two datasets, with no rate variation between sites. All other substitution-model and sampling parameters were set to the MrBayes 3.1.1 default values. Starting with random trees, two runs of 500,000 generations were used for each analysis, with samples taken every 100th generation. The first 25% of samples were discarded as burn-in.

Morphometrics

Plants from 26 populations were grown in a glasshouse in Oxford from 1 August 2003 to 1 October 2003. Seeds were sown in single-population trays, and seedlings were selected haphazardly within a few days of germination and transferred to individual pots. Plants were grown in eight randomized blocks, each population being represented once in each block. Blocks were rerandomized at intervals of four to five days. The final sample size was 179 plants.

The following morphological characters were measured for each plant (indices refer to leaf nodes on the main stem, with

the cotyledons denoted as node zero): plant height; dry mass; length of each of the first five internodes; length of petioles 2 to 4; length of leaf 3; width of leaf 3; length of branches 0 to 3; length of internodes 0 and 1; diameter of internode 1; length of stipules at node 5; peduncle length (if present); pedicel length; and the proportions of bi-, tri-, and tetralocular female flowers. Leaf areas and perimeters were calculated from the scanned images of five leaves per plant (from nodes one to five). The software package Shape (Iwata and Ukai 2002) was used for analysis of leaf shape. Elliptic Fourier descriptors were calculated from leaf perimeters and normalized by the longest axis, with some correction by eye. The symmetric coefficients were used in a principal component analysis (PCA), and principal component scores (PCS) for the first five components (those that each explained $> 0.5\%$ of the variation, 98.4% in total) were calculated for each leaf. These scores were then used as measures of leaf shape in later analysis (Iwata and Ukai 2002). Cluster analysis was used to identify ''natural'' groups; *z*-score-scaled population-mean morphological data were used in a clustering analysis, using within-group linkage of squared Euclidian distances. Cluster analysis was performed using SPSS (SPSS for Windows, release 11.0.0; SPSS Inc., Chicago, IL). A discriminant function analysis of this dataset is presented elsewhere (Obbard et al. 2006b).

Results

DNA Content and Ploidy

DNA content was highly correlated with ploidy amongst the annual mercuries, with diploid, tetraploid, and hexaploid samples of *M. annua* forming an almost linear series (Fig. 2). Diploid *M. annua* had a 4C DNA content between 2.62 pg and 2.65 pg (99% confidence interval of the grand mean). Tetraploid *M. annua* had a 4C value between 5.14 pg and 5.19 pg, while the hexaploid *M. annua* 4C value was between 7.73 pg and 7.80 pg. *Mercurialis huetii* had a 4C DNA content between 2.82 pg and 2.86 pg, significantly larger than diploid *M. annua* (Fig. 2). *Mercurialis canariensis* is tetraploid ($2n = 32$), but it had a significantly larger 4C value (6.43 pg to 6.46 pg, Fig. 2) than did tetraploid *M. annua*.

Intraindividual Variation in ITS

There was evidence for intraindividual variation in ITS sequences in both hexaploid *M. annua* and tetraploid *M. canariensis*. *Mercurialis annua*-specific primers amplified an ITS sequence from dioecious polyploid *M. canariensis* accessions that was closely related to but divergent from that of other *M. annua* (sequences labeled ''B'' in the polyploid *M. annua* clade, Fig. 3). *Mercurialis huetii*-specific primers amplified an additional ITS sequence identical to that found in *M. huetii* from hexaploid *M. annua* (sequences labeled ''B'' in the *M. huetii* clade, Fig. 3). Under the chosen reaction conditions, neither set of taxon-specific primers amplified a detectable product from controls containing only the alternative template (data not shown), and no *M. huetii*-like ITS sequence was amplified from tetraploid *M. annua*.

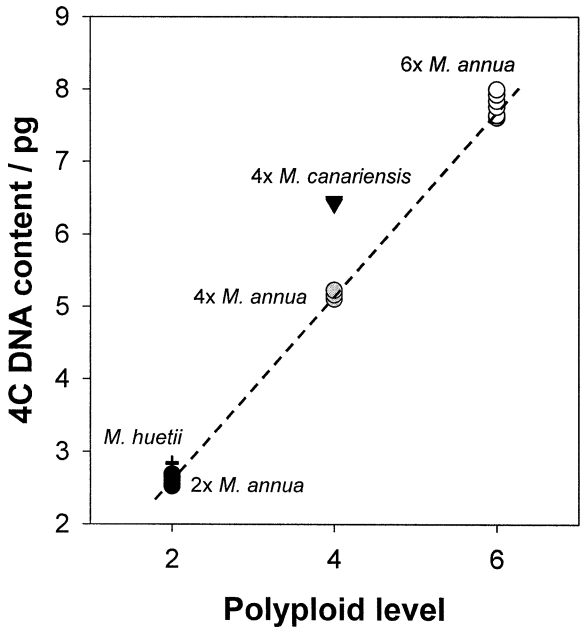

FIG. 2. Mean genome size (4C-value/pg) for the *Mercurialis* populations used in the morphometric analysis. Standard errors were all less than 0.02. All five labeled groups were significantly different from each other in DNA content (ANOVA and Tukey tests, $P < 0.001$ for each pairwise comparison). Although the DNA content of diploid, tetraploid, and hexaploid individuals of *M. annua* appears to form a linear series, both polyploid lineages have genomes smaller than would be expected based exclusively on summations of diploid *M. annua* (1–1.5% smaller), and/or *M. huetii* (4–10% smaller). *Mercurialis canariensis* has a much larger genome size than would be expected if it resulted exclusively from recent autopolyploidization within *M. annua* sensu lato.

ITS Phylogeny

The total aligned sequence, including the 5.8S rDNA, had a length of 774 bp. There were 102 parsimony-informative characters. There were no indels in the amplified 5.8S nrDNA sequences, and the only substitution in 5.8S nrDNA was shared by all the woody perennial species in the analysis (''woody perennials'' in Fig. 3). The absence of indels in the 5.8S region suggests that none of the sequences is evolving as a pseudogene. In the alignment of ITS1 and ITS2 there were 30 indels of 1–3 bp, one of 9 bp, and one of 13 bp; nine of these were parsimony informative. Although it does not affect any conclusions regarding sexual-system and polyploid evolution within the annual group, the alignment between *Mercurialis* species and the *Ricinus* outgroup was problematic in the spacer regions and thus ought to be treated with some caution.

The Bayesian consensus tree (both substitution models) and the strict consensus parsimony tree (of 12 equally most parsimonious trees) had identical topologies (see Fig. 3 for the Bayesian consensus tree). The gene tree inferred from ITS data indicates that the annual and woody perennial species of *Mercurialis* together form a monophyletic group, with *M. perennis* being sister to this clade (Fig. 3). However, the support for an annual clade was low (66% bootstrap/71% Bayesian posterior; ''annuals'' in Fig. 3), such that the relationship between the woody perennials, *M. canariensis* A

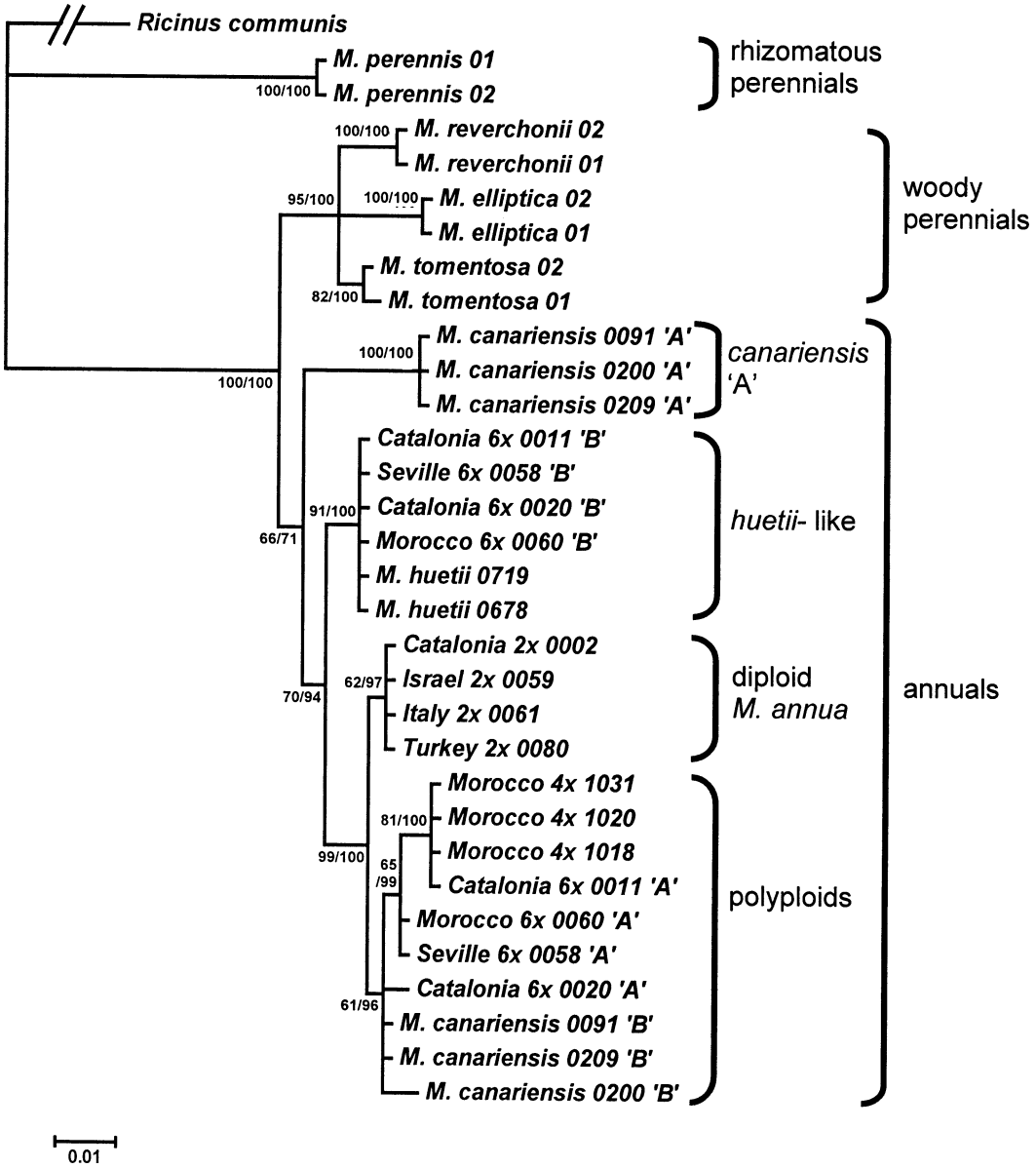

FIG. 3. Bayesian consensus tree based on the combined ITS1-5.8s-ITS2 dataset, using a single parameter model of nucleotide substitution with no rate variation between sites. All samples identified by source location and ploidy are *M. annua* sensu lato. Where two divergent ITS sequences were derived from the same individual, they are denoted ''A'' and ''B.'' Bootstrap support from 585 replicates of the maximum parsimony analysis (first) and Bayesian posterior support (second) are given above the respective node. Branch lengths are proportional to the inferred number of substitutions, except that of *Ricinus communis*, which has been shortened to clarify the remainder of the tree.

sequences, and other annuals is uncertain. Within the clade of annual species, the group comprising *M. huetii* and hexaploid *M. annua* ITS B sequences (''*huetii*-like'' in Fig. 3) were sister to a clade that includes diploid *M. annua*, tetraploid *M. annua*, and hexaploid A *M. annua* sequences. Note, however, that clade grouping the *M. annua* polyploids with *M. canariensis* B sequences received little bootstrap support (''polyploids'' in Fig. 3).

Chloroplast Phylogeny

The aligned lengths of the *trn*L-*trn*F and *mat*K-*trn*K sequences were 474 and 664 bp, respectively; in total there were 93 parsimony-informative characters. There were 26 indels in *trn*L-*trn*F dataset and 16 in the noncoding part of *mat*K-*trn*K; 13 of the cpDNA indels were associated with poly-T regions. In the combined cpDNA dataset, a total of 32 gap characters were parsimony informative. The amplification of *mat*K-*trn*K from *M. reverchonii* and *M. tomentosa* failed repeatedly, and these sequences were thus treated as missing data in all analyses. The Bayesian consensus tree (both substitution models) and the strict consensus of the four equally most parsimonious trees had identical topologies (Fig. 4). The phylogenetic positions of the two largest indels (91 bp and 106 bp) are marked by black arrows on Figure

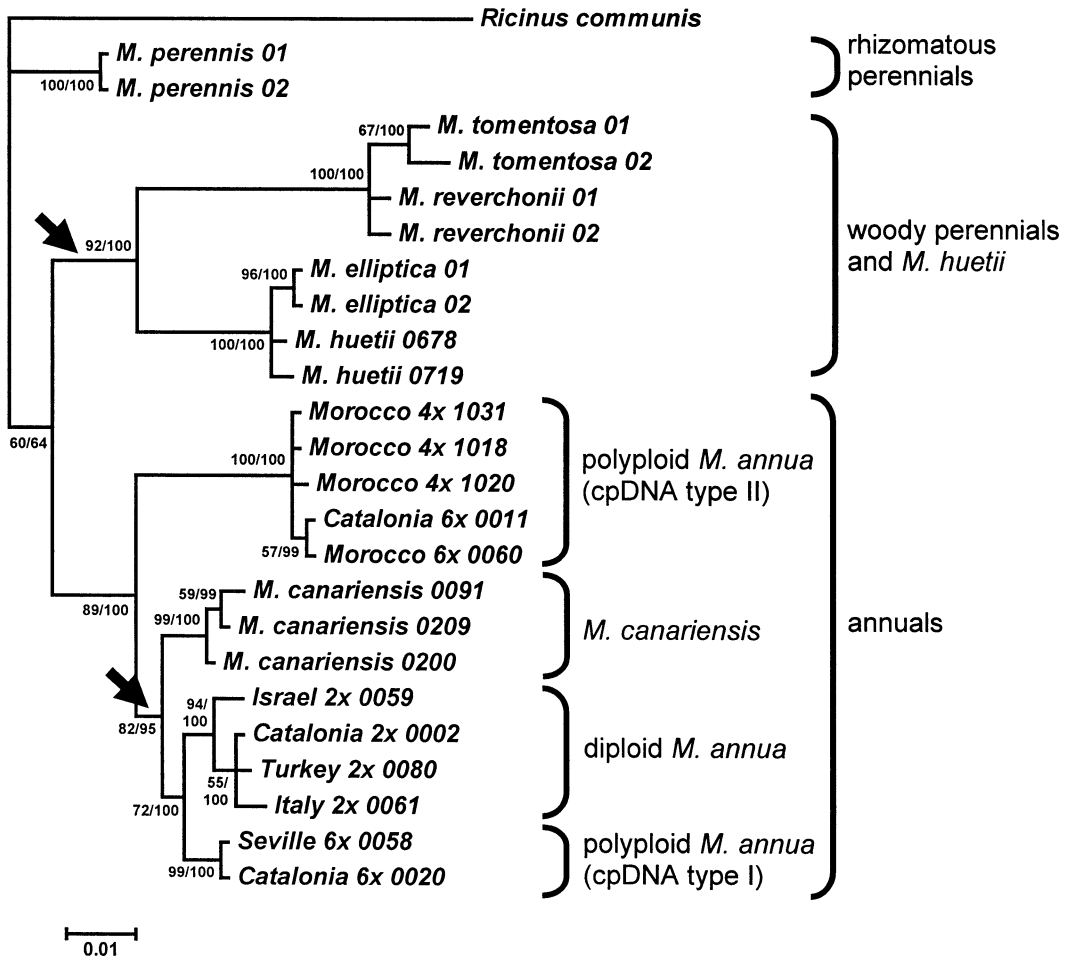

FIG. 4. Bayesian consensus tree based on the combined *trn*L-*trn*F *mat*K-*trn*K chloroplast datasets, using a single parameter model of nucleotide substitution with no rate variation between sites. All samples identified by source location and ploidy are *M. annua* sensu lato. Bootstrap support from 585 replicates of the maximum parsimony analysis (first) and Bayesian posterior support (second) are given above the respective node. Branch lengths are proportional to the inferred number of substitutions. Black arrows indicate the location of large indels (>90 bp). Although a single Type II cpDNA accession is presented for Iberian hexaploid samples, Type II chloroplasts were more common in Iberia than Type I (63% Type II, assayed by restriction digest; Obbard 2004), and Type I chloroplast sequences were not found in Morocco.

4. Each of three main clades (*M. perennis*, woody perennial species, and annual species; Fig. 4) were individually well supported (Bayesian posterior 100%). However, although the cpDNA analysis resolved the *M. perennis* clade as being sister to the other *Mercurialis* species in the sample, there was low support for the monophyly of the annuals and woody perennials (bootstrap 60%, posterior probability 64%; Fig. 4). Importantly, the Bayesian consensus tree inferred from chloroplast data differed from the ITS tree in the position of hexaploid *M. annua* and *M. canariensis* (compare Figs. 3 and 4), as well as of *M. huetii*, which was sister to *M. elliptica* within the woody perennial clade (Fig. 4).

Morphology

Despite the absence of discrete diagnostic characters in *M. huetii* (Durand and Durand 1985; Obbard et al. 2006b), both *M. huetii* and *M. canariensis* were morphologically distinct from *M. annua* in the clustering analysis of glasshouse-grown material (Fig. 5). Although there is little vegetative differentiation between diploid and polyploid *M. annua* in field-grown samples, glasshouse-grown material revealed significant vegetative differences; for example, polyploids were about 10% taller ($P < 0.001$) and possessed rounder leaves (*t*-test on the first principal component scores of leaf shape: $P < 0.001$). These differences were reflected in the separation of diploids from polyploids by the clustering analysis. In contrast, clustering analysis could not separate tetraploid from hexaploid *M. annua*, which can be distinguished only by means of chromosome counts or DNA content.

DISCUSSION

Phylogenetic, morphological, and genome size analyses indicate that the evolution of ploidy and sexual-system diversity in the clade of annual species in the genus *Mercurialis* has been complex. In particular, the loose association between ploidy and sexual systems previously documented in the genus (Thomas 1958; Durand 1963; Durand and Durand 1992) would appear to have been molded by a combination

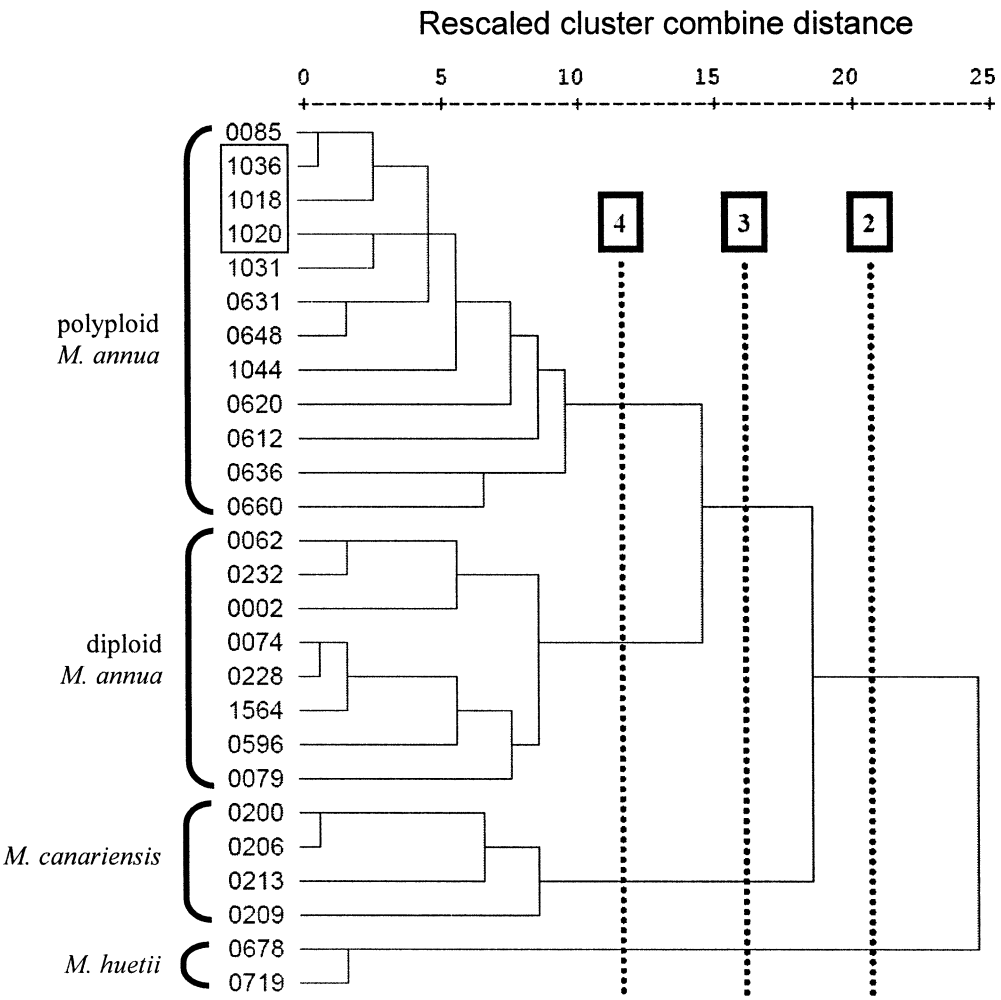

FIG. 5. Hierarchical clustering dendrogram of scaled morphological data, calculated as means over populations. Clustering was based on average within-group linkage, using squared Euclidian distances calculated from 55 *z*-score-transformed vegetative characters (sample sizes of five to eight individuals per population). The three most informative classifications divide the dataset into two, three, or four distinct groups, respectively (marked with dotted lines). The most divisive classification identifies *M. huetii*, *M. canariensis*, diploid *M. annua*, and polyploid *M. annua* populations as distinct clusters. No clear morphological distinction between tetraploid (marked by box) and hexaploid *M. annua* could be identified.

of homoploid speciation, genome duplication, and hybridization. Below we discuss the diversification of the annual *Mercurialis* clade with references to (1) evidence for hybridization and a combination of auto- and allopolyploidy, (2) introgression and capture of chloroplast haplotypes both within the annual clade and from lineages outside of it, and (3) evidence from our analysis of genome size and vegetative morphology. Finally, we discuss the possible significance of polyploidy and hybridization for the diversification of sexual systems in *M. annua*. In particular, we consider the role that hybridization may have played in the evolution of the otherwise rare sexual system, androdioecy.

Hybridization and Allopolyploidy

The ITS and chloroplast gene trees (Figs. 3 and 4) broadly support the hypothesis that the perennial taxa of *Mercurialis* are sisters to the annual taxa. They also identify considerable differentiation within the *M. annua* complex. Importantly, we found within-individual variation in the ITS sequences in both *M. canariensis* and hexaploid *M. annua*. The fact that these sequence variants cluster in different parts of the gene trees suggests a hybrid origin for both lineages. This hypothesis is consistent with both morphological analysis and genome size data. Additionally, the unexpected placement of plastid sequences from *M. huetii* suggests that recent long-range chloroplast capture has occurred between the woody perennials and the annuals (Fig. 4). Previously, ploidy variation in the *M. annua* species complex has been attributed exclusively to autopolyploidy (Durand and Durand 1985, 1992; Krähenbühl et al. 2002). Our data, however, reveal a more complex evolutionary history involving both auto- and allopolyploidy (see Fig. 6).

The origin of M. canariensis

The *M. canariensis* plastid genome is markedly divergent from that represented by both of the two *M. annua* s.l. chlo-

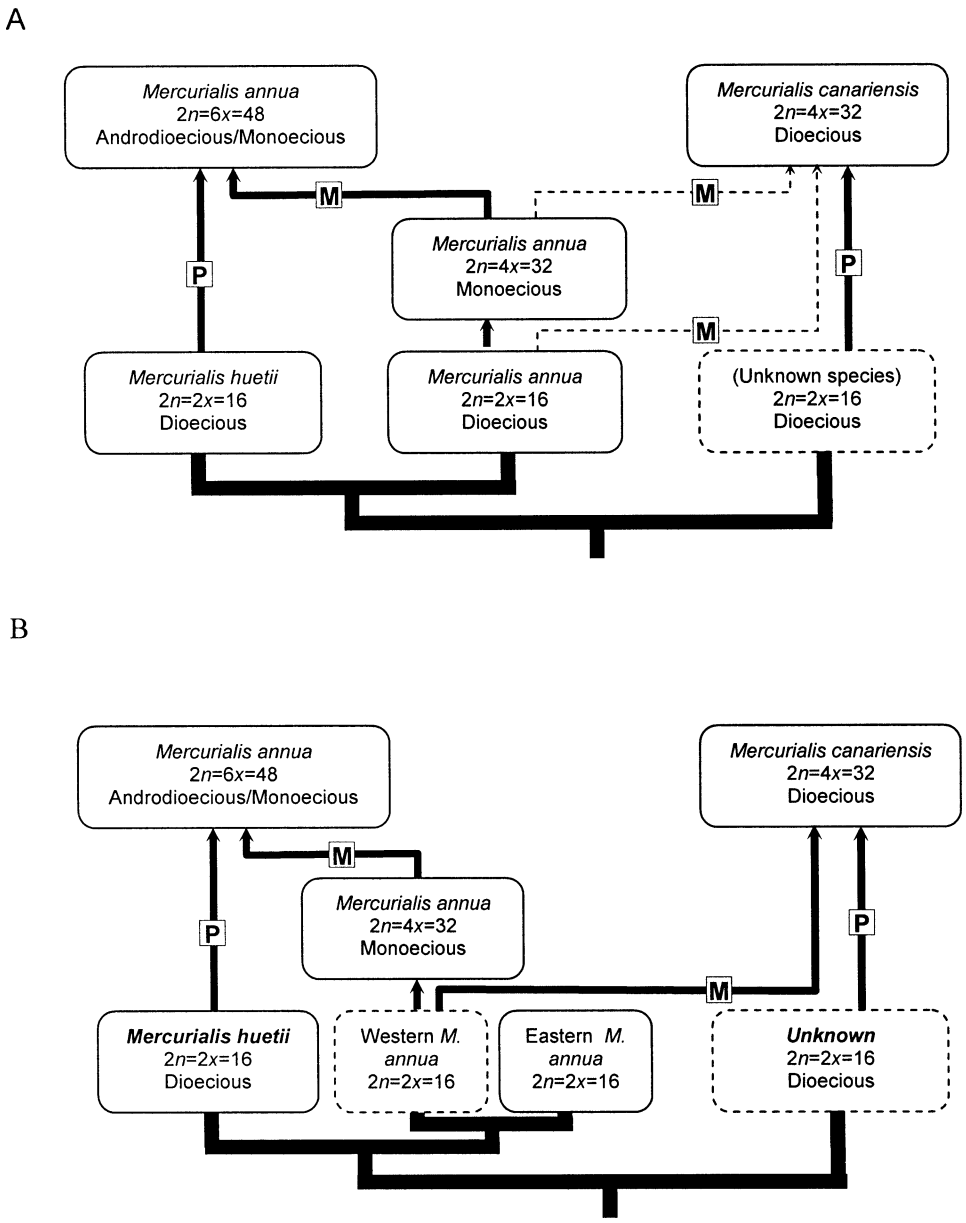

FIG. 6. Hypotheses for the relationships between the annual lineages of *Mercurialis*. Heavy lines indicate the phylogenetic relationships between diploid species, and thin arrows show polyploidization and/or hybridization events; M indicates proposed maternal parentage, and P indicated proposed paternal parentage. (A) Diploid *M. annua* proposed as the parent of polyploid *M. annua*. The heterogeneous ITS types present in hexaploid *M. annua* show that it has an allopolyploid origin through hybridization between *M. annua* and *M. huetii*, and the hexaploid chromosome complement is consistent with hybridization between a tetraploid and a diploid lineage, followed by chromosome doubling. ITS data also show *M. canariensis* to be allopolyploid in origin, probably a hybrid between *M. annua* sensu lato and an unknown taxon (dashed box). Chloroplast sequence similarity to *M. annua* sensu lato suggests that *M. annua* sensu lato was the maternal parent, and chromosome numbers are consistent with both parents being diploid. (B) To account for the greater similarity between ITS sequences from *M. canariensis* and polyploid *M. annua*, we propose that the diploid progenitor of these taxa may have been an earlier western lineage of *M. annua*, divergent from the lineage that has recently expanded from the east (Obbard et al. 2006a).

roplast clades (Fig. 4). Moreover, two distinct ITS sequences are identifiable within each *M. canariensis* individual (Fig. 3). One of these is the most divergent of the sequences derived from the annuals (A sequences in Fig. 3) and is unlike any previously identified *Mercurialis* species (i.e., those examined here or by Krähenbühl et al. 2002); the other falls inside the *M. annua* s.l. clade (B sequences in Fig. 3). These data strongly implicate a hybrid origin for *M. canariensis*, with one ITS sequence likely derived from a parent in the *M. annua* clade and the other from an unknown lineage sister to the extant annual species (''Unknown species'' in Fig. 6). Because *M. annua* displays maternal chloroplast inheritance (Obbard 2004), the plastid sequences implicate a member of the *M. annua* clade as the maternal parent and the unknown lineage as the paternal contributor (M and P in Fig. 6A).

The hypothesis of an allopolyploid origin for *M. canar-*

iensis is also supported by genome size measurements and morphology. Specifically, the genome of the tetraploid *M. canariensis* is 26% larger than would be expected from a summation of two diploid *M. annua* genomes, and 18% larger than a combination of diploid *M. annua* and *M. huetii* (Fig. 2). Therefore, unless there has been a considerable increase in DNA content, *M. canariensis* is unlikely to have been derived from hybridization or polyploidization exclusively among the other known annual species. The morphology of *M. canariensis* is also distinctive (Fig. 5, and see Obbard et al. 2006b): plants are larger and more robust than the related species, inflorescences are branched, stipules are large and recurved, clusters of male flowers are subtended by a bract, and female flowers are often trilocular (Obbard et al. 2006b). Many of these characters are present elsewhere in the genus amongst the woody perennials (e.g., more robust growth in the perennial *M. reverchonii*, large stipules in *M. tomentosa*). We therefore speculate that they represent characters derived from the unknown parent.

Of course, it remains an open question whether diploid or tetraploid *M. annua* s.l. acted as the maternal parent of *M. canariensis* (dashed arrows, Fig. 6A). On the one hand, the plastid tree groups *M. canariensis* most strongly with the clade containing diploid *M. annua* and type I hexaploid chloroplasts. On the other hand, ITS B sequences group *M. canariensis* more closely to the majority of polyploid *M. annua*. Because *M. canariensis* has a chromosome count of $2n = 32$, and the base chromosome number of the genus is $x = 8$ (Krähenbühl et al. 2002), it is more parsimonious to invoke allopolyploid hybridization between two diploid parents than a scenario involving parentage with higher ploidy levels and subsequent chromosome loss.

To account for both the plastid and ITS sequence data, it is implausible that the extant diploid *M. annua* lineage contributed directly to *M. canariensis*. In addition, phylogeographic data indicate that diploid *M. annua* only recently migrated into Europe from the eastern Mediterranean Basin (Obbard et al. 2006a), further making it unlikely as a parent. It therefore seems probable that the origin of *M. canariensis* dates from an earlier occupation of the western Mediterranean by a diploid lineage that was more closely related to modern *M. annua* than is *M. huetii* (Fig. 6B). In light of the high rate of endemism and known occurrence of relictual species within the Canary Islands (e.g., Francisco-Ortega et al. 1996; Juan et al. 2000), the unknown parental taxa, or *M. canariensis* itself, may have persisted for an extended period on Tenerife.

Evolution of M. annua sensu lato

Our finding of *M. huetii*-like ITS sequences in hexaploid *M. annua* individuals (Fig. 3) implicates a hybrid origin for hexaploid *M. annua*. Based on plastid sequence similarity, it is most likely that *M. annua* s.l. acted as the maternal parent and *M. huetii* as the paternal parent (Fig. 6). The apparent discrepancy between our findings and those of Durand and Durand (1985, 1992) and Krähenbühl et al (2002), who both suggested an exclusively autopolyploid series, is probably due to our improved ability to identify divergent ITS sequences through the use of specific primers (Rauscher et al. 2002; Alvarez and Wendel 2003).

Interestingly, we failed to find any evidence of *M. huetii*-like ITS sequences in tetraploid *M. annua* individuals. This suggests that tetraploid *M. annua* may indeed be an autopolyploid, as previously proposed (Durand 1963). The ITS A sequences from hexaploid *M. annua* cluster with tetraploid *M. annua*, and are separated from diploid *M. annua* (Fig. 3), so that tetraploid *M. annua* may have acted as a parent to the hexaploids (Fig. 6). This idea is broadly supported by chromosome numbers, with hexaploids being the result of hybridization between a diploid and a tetraploid lineage, yielding a triploid, followed by genome duplication.

Given that extant populations of tetraploid and hexaploid *M. annua* occur in the western Mediterranean Basin in Iberia and Morocco (Fig. 1), and that diploid and polyploid *M. annua* ITS sequences are highly divergent, it is plausible that a distinct lineage of diploid *M. annua* acted as the parent of autotetraploid *M. annua*. This scenario agrees with that outlined above for *M. canariensis* (Fig. 6B), which similarly proposes a divergent diploid *M. annua* lineage at the western end of the Mediterranean. However, we have no direct evidence for such a lineage, and we cannot exclude an alternative hypothesis in which tetraploid *M. annua* originated at the eastern end of the Mediterranean and then migrated west.

In polyploid *M. annua*, genome size measurements are consistent with both an auto- and an allopolyploid origin. Thus, the 4C genome for diploid *M. annua* is approximately half the size of that of tetraploid *M. annua* (i.e., 2.63 pg vs. 5.20 pg, Fig. 2) and a third the size of that of hexaploid *M. annua* (7.77 pg). In each case the observed genome size is slightly smaller than expected from a summation of diploid genomes, suggesting the loss of DNA following polyploidization, as is commonly observed (Bennett et al. 2000; Wendel et al. 2002). Importantly, because the genome sizes of *M. huetii* and diploid *M. annua* are so similar (Fig. 2), our data are also consistent with an allopolyploid origin for hexaploid *M. annua* (Fig. 6), in which genome size for the newly synthesized polyploid would be in the range of 7.96–8.05 pg. This would only entail a loss of around 3% of the genome.

Our morphometric analysis of individuals grown in the glasshouse uncovered little vegetative differentiation within the *M. annua* s.l. complex (Fig. 5; see also Obbard et al. 2006b). In particular, there was no clear vegetative distinction between tetraploid and hexaploid *M. annua*. This is perhaps surprising, given our hypothesis that tetraploid *M. annua* comprises four copies of the same genome whereas hexaploids possess an additional two copies of the divergent *M. huetii* lineage. However, morphological differentiation between all members of the *M. annua* s.l. complex and *M. huetii* is small (Durand 1963; Obbard et al. 2006b), with *M. annua* being only slightly larger and less branched than *M. huetii* when grown under controlled conditions.

Introgression and chloroplast capture

The phylogenetic position of plastid DNA sequences amplified from *M. huetii*, along with those amplified from a minority of hexaploid *M. annua* (cpDNA type I), remain unexplained in the scheme outlined in Fig. 6. In particular, *M. huetii* possesses a highly divergent cpDNA type that clusters closely with *M. elliptica*, within the well-supported clade of

woody perennials (Fig. 4). We propose this may be the result of the recent introgression of chloroplast sequences through hybridization between *M. huetii* and *M. elliptica*, which are sympatric in northern Iberia (Güemas 1997). Given that hybridization within the genus is common (Pax 1914; Krähenbühl et al. 2002), this hypothesis certainly seems plausible. Similarly, the occurrence of Type I chloroplasts in about 40% of Iberian hexaploid *M. annua* individuals, but not in Moroccan polyploids (Obbard 2004), may represent a relatively recent case of introgressive chloroplast capture from diploid *M. annua* (Fig. 4) following hybridization (Rieseberg and Soltis 1991; Tsitrone et al. 2003). Indeed hybridization between diploid and hexaploid *M. annua* appears common, and although hybrid fertility is very low, hybrids with a diploid maternal parent have higher fertility than those with a hexaploid maternal parent, potentially providing an explanation for the observed unidirectionality of chloroplast introgression (Buggs and Pannell 2006).

Caveats

The hybridization scheme presented above seems the best interpretation of the available data. However, sampling in some groups was limited, and it is possible that additional sampling would alter our conclusions. For example, more exhaustive sampling of polyploid populations of *M. annua* might reveal hexaploid individuals lacking *M. huetii*-like ITS sequences or tetraploid individuals possessing *M. huetii*-like sequences. In either case, such a finding would weaken support for a hybrid origin of hexaploid *M. annua*. Similarly, support for a chloroplast-capture origin for the Type I chloroplast of hexaploid *M. annua* would be eroded if wider sampling revealed this cpDNA type in polyploid populations in Morocco (current sample comprises 57 individuals across 10 populations; Obbard 2004). Notwithstanding these caveats, it should be noted that accessions were sampled both on the basis of the results of an earlier isozyme survey (about 100 populations; Obbard 2004; Obbard et al. 2006b) and to maximize the geographic range represented in each group. We therefore think it unlikely that our interpretation of a hybrid origin for hexaploid *M. annua* and tetraploid *M. canariensis* will be altered by further sampling.

Implications for Sexual-System Evolution

Our study supports the conclusion of earlier work (Durand and Durand 1985; Krähenbühl et al. 2002) that monoecy is derived from dioecy in *Mercurialis*. However, evolution in *Mercurialis* has evidently been more complex than previously thought (Durand and Durand 1985, 1992; Krähenbühl et al. 2002), and the complexities (involving both autopolyploidy and allopolyploid hybridization; Fig. 6) have interesting implications for sexual-system evolution in the genus, particularly the origin of the androdioecy, which is difficult to evolve de novo (reviewed in Pannell 2002).

The evolution of monoecy from dioecy

Autopolyploidization may have precipitated the evolution of monoecy from dioecy in *M. annua* in two ways. First, genome duplication in itself might have disrupted the sex-determination mechanism in a formerly dioecious population, so that monoecious individuals began segregating in neopolyploids prior to any selection for or against a strategy of combined sexes (Westergaard 1958; Pannell et al. 2004). Indeed, Durand (1963) found monoecious individuals in the F_2 progeny of artificial autotetraploids that had been generated from diploid dioecious individuals of *M. annua*, with phenotypes similar to those found in natural populations. Second, selection on neopolyploids might be expected to favor individuals capable of selfing (reviewed in Pannell et al. 2004). Thus, because self-compatible neopolyploid individuals need not cross with a lineage of different ploidy, it is predicted that they will avoid the costs associated with the sterility of interploidy hybrids through minority cytotype exclusion (Levin 1975; Rausch and Morgan 2005). Recent experiments have shown this effect to be strong in *M. annua*, particularly at low densities (Buggs and Pannell 2006). Polyploidy may also reduce the level of inbreeding depression in neopolyploids below the threshold required to prevent the automatic spread of self-fertilization (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Ronfort 1999). Finally, an ability to self-fertilize, conferred by monoecy, is expected to enhance the potential of new polyploids to spread into areas of marginal habitat (Stebbins 1950; Brochmann et al. 2004; Pannell et al. 2004). Nevertheless, although these factors may have been significant in the success of monoecious polyploids in *M. annua*, dioecy is also widespread in polyploid lineages in the genus in general (Krähenbühl et al. 2002), as well as in *M. canariensis*, described here.

The evolution of androdioecy

The proposed allopolyploid origin of hexaploid *M. annua* raises the interesting possibility that androdioecy in this lineage evolved as a direct result of hybridization. Androdioecy is known to be difficult to evolve from hermaphroditism or monoecy through the spread of a female-sterility mutation, because the resulting males must be able to sire more than twice the progeny of hermaphrodites for them to succeed (Lloyd 1975; Charlesworth and Charlesworth 1978; Charlesworth 1984; Pannell 2002); this requirement would seem prohibitive for a new female-sterile individual. However, the *M. annua* allohexaploid lineage may have inherited a fully functional monoecious phenotype from its tetraploid parent as well as genes required for a specialized male phenotype, including specialized inflorescence morphology (erect peduncles) not seen in tetraploid *M. annua*, from the *M. huetii* ancestor. This would thus sidestep a need for the re-evolution of a specialized male inflorescence de novo.

Of course, the conjecture that androdioecy in *M. annua* may have been precipitated by hybridization remains speculative. At least two other scenarios also seem plausible. First, our hypothesis presupposes that the male allocation and architecture in hexaploid *M. annua* are the result of the expression of genes derived from dioecious *M. huetii*, but it is possible that the genes or regulatory regions in question are in fact those from the tetraploid *M. annua* genome. Currently, these genes are not expressed to produce a male phenotype in tetraploids, but it is possible that they were expressed, or expressed differently, in the tetraploid background in the past,

and that they have since been reactivated in the hexaploid lineage. These alternative scenarios might be distinguished by means of molecular evolutionary analysis, and/or analysis of transcripts of genes involved in sex determination and expression in the respective lineages (Ainsworth 2000; Khadka et al. 2005). A second possibility is that androdioecy evolved in hexaploid *M. annua* from dioecy, with the spread of modified females that produced some pollen; such modified females would enjoy the advantage of reproductive assurance under mate or pollen limitation; for example during colonization (Pannell 2001; Wolf and Takebayashi 2004). It is difficult to reject this scenario, but the current distribution of sexual systems in *M. annua*, particularly the absence of dioecy in hexaploid *M. annua*, provides no support for it.

Conclusions

The annual species of the genus *Mercurialis* continue to provide an exceptional model system for study of the evolution and maintenance of combined versus sexual sexes (Camerarius 1694; Yampolsky 1919; Durand and Durand 1985, 1991; Pannell 1997a; Krähenbühl et al. 2002; Pannell et al. 2004; Khadka et al. 2005). They join ranks with only a handful of species in which both dioecy and functional hermaphroditism occur in a single species complex (e.g., Costich and Meagher 1992; Dorken and Barrett 2003; Case and Barrett 2004), and they present one of the very few examples of androdioecy, a potential intermediate path between sex-allocation extremes (Charlesworth and Charlesworth 1978). Previous work on *M. annua* has considered selection on the sexual system acting both within populations (Pannell 1997c) and in metapopulations (Pannell 1997b,c; 2001). Other work has shown that the current distribution of sexual systems needs to be understood within the phylogeographic history of the respective lineages, which have recently come together in the Iberian Peninsula from two different Pleistocene refugia (Obbard et al. 2006a). The present study now places the variation in sexual systems in *Mercurialis* within a phylogenetic context. The evolutionary history of these lineages has clearly been complex and reticulate, involving autopolyploidy, allopolyploidy, and introgressive chloroplast capture. Answers to how these processes have influenced the remarkable sexual variation in the group await the analysis of the relevant genomes and patterns of gene expression.

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