Published in Plant Systematics and Evolution 234, issues 1-4, 155-169, 2002
which should be used for any reference to this work

Chromosome and breeding system evolution of the genus *Mercurialis* (Euphorbiaceae): implications of ITS molecular phylogeny

M. Krähenbühl[1], **Y.-M. Yuan**[1,2], and **P. Küpfer**[1]

[1]Institut de Botanique, Laboratoire de botanique évolutive, Université de Neuchâtel, Suisse
[2]MOE Key Lab for Biodiversity Sciences & Ecological Engineering, Beijing Normal University, Beijing, P. R. China

Abstract. The internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA were amplified and sequenced from 19 samples representing all species of the genus *Mercurialis* and two outgroup species, *Ricinus communis* and *Acalypha hispida*. The length of ITS1 in the ingroups ranged from 223 to 246 bp and ITS2 from 210 to 218 bp. Sequence divergence between pairs of species ranged from 1.15% to 25.88% among the ingroup species in the combined data of ITS1 and ITS2. Heuristic phylogenetic analyses using Fitch parsimony on the combined data of ITS1 and ITS2 with gaps treated as missing generated 45 equally parsimonious trees. The strict consensus tree was principally concordant with morphological classification. Within the genus, the ITS sequences recognised two main infrageneric clades: the *M. perennis* complex including three Eurasian stoloniferous species (*M. leiocarpa*, *M. ovata* and *M. perennis*) and the western Mediterranean group including eight both annual and perennial species. Of the western Mediterranean clade, the annual and perennial species grouped respectively into two different groups, and the annual life form is revealed as a synapomorphic character derived from perennial, whereas in the Eurasian clade ITS phylogeny suggested *M. leiocarpa* as basal clade sister to *M. perennis* and *M. ovata*. ITS phylogeny failed to resolve the relationships among the different cytotypes of *M. ovata* and *M. perennis*. ITS phylogeny also suggested rapid karyotypic evolution for the genus. The karyotypic divergence among the perennial species of western Mediterranean region did not corroborate the nucleotide sequence divergence among the species. Optimisation of chromosome numbers onto the ITS phylogeny suggested $x = 8$ to be the ancestral basic chromosome number of the genus. ITS phylogeny confirmed that the androdioecy of *M. ambigua* is derived from dioecy. The nucleotide heterozygosity and additivity in ITS sequences clearly confirm the interspecific hybridisation in the genus *Mercurialis*.

Key words: Euphorbiaceae, *Mercurialis*, ITS, phylogeny, hybridisation, chromosome, breeding system, evolution.

Introduction

The genus *Mercurialis* (Euphorbiaceae) comprises 10 species mostly distributed in the areas of the western Mediterranean basin. The three annual species *M. annua* L., *M. huetii* Hanry, and *M. ambigua* L. fil. are sometimes lumped into *M. annua* L. in broad sense (Tutin et al. 1968), while *M. ambigua* was sometimes fur-

ther split into *M. ambigua* s. str. and *M. monoica* (Moris) Durand based on different sexual expressions and polyploid levels (Durand and Durand 1992). Here we do not follow this splitting, and consider the different monoecious polyploid types as *M. ambigua*. While *M. ambigua* and *M. huetii* are limited to the western Mediterranean areas, *M. annua* is widely spread in most part of Europe and further naturalised in North America. Among the perennial species, two groups of species can be recognised. One group consists of the rhizomatous species *M. perennis* L., *M. ovata* Sternb. et Hoppe, and *M. leiocarpa* Sieb. et Zucc. which are usually growing in shady places or in woods. *M. perennis* is widespread in most part of Europe. *M. ovata* is found in central to southeastern Europe. Only *M. leiocarpa* occurs in Asia (eastern Himalayas, southern China, South Korea, and Japan). The another group consists of the rest four perennial species, *M. corsica* Cosson, *M. elliptica* Lam., *M. reverchonii* Rouy, and *M. tomentosa* L., all of them are non-rhizomatous and are limited to the western Mediterranean basin occupying woodland, shadowed, sandy or rocky places. Except for some taxonomic treatments in different floras (Tutin et al. 1968, Valedes et al. 1987), no phylogenetic consideration has been made on this genus. Most recent studies concern its karyology and breeding systems (Krähenbühl 1984, Krähenbühl and Küpfer 1995, Durand and Durand 1992, Pannell 1997).

Chromosome numbers have been counted for all the species from different regions. Wide range of chromosome number variation (including polyploidy, dysploidy, B chromosomes etc.) is documented among the species and within some species (Krähenbühl 1984, Krähenbühl and Küpfer 1995, and unpublished data). While *M. annua* and *M. huetii* remain diploid ($2n = 16$), chromosome numbers of $2n = 32$, 48, 80, 96, and 112 have been recorded for *M. ambigua* (including *M. monoica*). Autopolyploidy was suggested as the cause of karyological evolution in these annual species (Durand and Durand 1992). Among the perennial species, chromosome numbers of $2n = 48$, 64, 80, 96, 112 and different aneuploid numbers have been recorded for *M. perennis*. The numbers $2n = 16$ and 32 have been found from *M. ovata* and $2n = 16$ and 48 from *M. leiocarpa* among the perennial species. While the above species showed the basic number of $x = 8$, other basic numbers were also revealed, for example, *M. corsica* with $2n = 66$ ($x = 33$), *M. elliptica* with $2n = 42$ ($x = 21$) and $2n = 220$ ($x = 10$ or 11?), *M. reverchonii* with $2n = 26$ ($x = 13$), and *M. tomentosa* got $2n = 26$ ($x = 13$) (Krähenbühl unpublished data). Allopolyploidy arisen from hybridisation of different cytotypes has been proposed to explain the cytogeographic and cytogenetic variations in perennial species (Krähenbühl 1984, Krähenbühl and Küpfer 1995). Inter-specific hybridisation has been previously suggested in this genus (Ascherson and Graebner 1913, Pax and Hoffmann 1914, Tutin et al. 1968). Our karyological observations on some spontaneous offspring generated from the transplanted plants in Neuchâtel botanical garden have revealed new chromosome numbers not found in any wild parental species (unpublished data). For example, some individuals were found with $2n = 34$, a possible hybrid between *M. elliptica* ($2n = 42$) and *M. reverchonii* ($2n = 26$). Some individuals were found with $2n = 54$, a putative hybrid between *M. elliptica* ($2n = 42$) and *M. corsica* ($2n = 66$). Both gross morphology and chromosome numbers suggest interspecific hybridisation.

The sexual systems expressed in this genus have attracted recent research interests (Durand and Durand 1992, Pannell 1997). While most species of *Mercurialis* are exclusively dioecious (i.e. all genets in a population are either male or female, e.g. *M. tomentosa*, *M. elliptica*), some species are monoecious (i.e. a single genet has both male and female functions but the male and female functions are performed separately in different flowers, e.g. *M. leiocarpa*). Still some species have both monoecious and male individuals co-existing in a population (e.g. *M. ambigua*). Here we consider such situation as functional androdioecy following Pannell (1997). Androdioecy

in the strict sense refers to the situation where male and hermaphrodite genets with perfect flowers coexist in a population (Richards 1997), and is a rare phenomenon only proven in *Datisca* (Liston et al. 1990). Durand and Durand (1992) inexactly described the sexual expression of *M. ambigua* as andromonoecy that should refer to a hermaphrodite bearing both male and hermaphrodite flowers (Richards 1997). Pannell (1997) considered the coexistence of both male and monoecious individuals in *M. ambigua* (included in *M. annua*) as functional androdioecy. Dioecy was suggested as the ancestral state, and both monoecy and androdioecy were thought to be derived from dioecy in *Mercurialis* (Durand and Durand 1992, Pannell 1997). Karyological observations revealed that at least in the annual species higher polyploid levels correlated with the tendency toward androdioecy.

However, the ancestral conditions of the basic number $x = 8$ and dioecy have never been explicitly examined using a phylogenetic approach, and thus remain untested. A well-corroborated phylogeny of the genus, based on characters independent from chromosome numbers and breeding systems, becomes necessary to test the karyological and observational conclusions. Thus we reconstructed the phylogeny of the genus based on the sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA. The ITS sequence has proven to be a valuable source of characters to address phylogenetic relationships among closely related species in different plant families (Baldwin 1992, 1993; Baldwin et al. 1995; Yuan and Küpfer 1995; Yuan et al. 1996; Wendel et al. 1995; Vargas et al. 1999; Panero et al. 1999; Ballard and Sytsma 2000; Schmidt and Schilling 2000; Francisco-Ortega et al. 2001). The ITS sequences have also proven powerful in revealing hybridisation and reticulate evolution (e.g. Sang et al. 1997). In the present study, we reconstructed the ITS phylogeny of all the species of *Mercurialis* to address the following particular problems: (1) if $x = 8$ represents the ancestral state in chromosome evolution, (2) if monoecy and androdioecy are derived from dioecy in breeding system evolution, and (3) if there is any molecular documentation for the possible interspecific hybridisation in the genus *Mercurialis*.

Materials and methods

Plant materials and DNA extraction. The species analysed, and their origins and chromosome numbers are given in Table 1. For some dioecious species, both male and female individuals were sampled separately to reveal any possible variation between them. Leaves were collected from a single individual growing in the botanical garden or from a single individual of dried herbarium material. All voucher specimens were deposited in the herbarium of the University of Neuchâtel (NEU). In the most recent classification of Euphorbiaceae (Webster 1994), *Merurialis* was placed in the tribe Acalypheae and its most closely related taxa should be the two small genera, *Seidelia* Baillon and *Leidesia* Muell., from South Africa (all belong to the same subtribe Mercurialinae as *Mercurialis*). Unfortunately, both genera were unavailable to us. So we chose two other species, *Ricinus communis* L. of the subtribe Ricininae and *Acalypha hispida* Burm. f. of the subtribe Acalyphinae, as outgroups, although their phylogenetic relationships with *Mercurialis* have never been explicitly addressed. Total DNAs were extracted from about 2 g of fresh or 100 to 300 mg of dried leaves pulverised in liquid nitrogen with hot 2x CTAB buffer according to the protocol of Doyle and Doyle (1987).

Amplification of ITS region and the primers used. A standard double-strand polymerase chain reaction (PCR) was used to amplify the entire ITS region, using primers ITS5 and ITS4 (White et al. 1990). The same primers and two more internal primers, ITS2 and ITS3 (White et al. 1990), were also used in subsequent sequencing. A minor modification in ITS5 was made. The modified sequence of the primer ITS5 is 5′-GGAAGTAGAAGTCGTAACAAGG-3′. All of these primers were purchased from Microsynth (Switzerland).

PCRs were carried out in 25-μl reaction mixtures containing 16.88 μl of sterile double-distilled water, 2.5 μl of 10x Taq polymerase reaction buffer, 1 μl equimolar of 5mM dNTP, 1.25 μl each of 10 μM primer ITS5 and ITS4, 0.125 μl of 5 Unit/μl Taq DNA polymerase

Table 1. The origins of samples, their chromosome numbers and other characters analysed for the genus *Mercurialis* and the outgroups

Species	Coll. No.	Locality	Life form[1]	Sex[2]	2n
M. ambigua L.	M91-092-10	Gonnosfanadiga, Sardegna, Italy	A	B	62
M. ambigua L.	M91-093	Gonnosfanadiga, Sardegna, Italy	A	B	80
M. annua L.	M94-1	Neuchâtel (NE), Switzerland	A	M	16
M. annua L.	M94-2	Neuchâtel (NE), Switzerland	A	F	16
M. corsica Cosson	M89-282-03	Col de S. Stefano, Corse, France	P	F	66
M. elliptica Lam.	M89-296-07	Hinojos, Almonte, Huelva, Spain	P	M	42
M. elliptica × corsica	M90-286-02	Botanical Garden, Neuchâtel, Switzerland	P	F	54
M. elliptica aff.	M91-124	Ourika, Iraghf, Morocco	P	M	220
M. huetii Hanry	M87-341-01	Pic St-Loup, Tréviers, France	A	F	16
M. leiocarpa Sieb. et Zucc.	M92-068-01	Kunming, Yunnan, China	P	B	16
M. ovata Sternb. et Hoppe	M89-348-01	Vicinity of Istanbul, Turkey	P	M	32
M. ovata Sternb. et Hoppe	M89-353-02	Erymanthos, Greece	P	M	16
M. perennis L.	M94-3	Neuchâtel (NE), Switzerland	P	F	48
M. perennis L.	M94-4	Chaumont (NE), Switzerland	P	M	64
M. perennis L.	M94-5	Chaumont (NE), Switzerland	P	F	64
M. perennis L.	M94-6	Ramsei (BE), Switzerland	P	F	80
M. perennis L.	M94-7	Ramsei (BE), Switzerland	P	M	80
M. perennis L.	M94-8	Sicile, Italy	P	F	96
M. reverchonii Rouy	M89-305-10	Algeciras, Cádiz, Spain	P	M	26
M. reverchonii × elliptica	M91-005-11	Botanical Garden, Neuchâtel, Switzerland	P	F	34
M. tomentosa L.	M87-057-01	Congues-sur-Orbiel, Aude, France	P	F	26
Acalypha hispida Burm. f.	M94-9	Cultivated in Pappiliorama, Neuchâtel, Switzerland	P	B	?
Ricinus communis L.	M94-10	Cultivated in Botanical Garden, Neuchâtel, Switzerland	P	B	20

[1] A refers to annual, and P to perennial

[2] B refers to cosexual (monoecious), M to male, F to female

(Appligene, Illkirch, France), and 2–8 ng (2 μl of 1–4 ng/μl) genomic DNA. PCR was performed on Perkin-Elmer GeneAmp 2400 thermal cycler with heated cover under the following conditions: one cycle of 2 min at 94 °C linked to 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. Samples were held for a final 5 min at 72 °C to complete primer extension.

Purification of PCR products and DNA sequencing. Five microliters of each PCR reaction were run on a 1.0% agarose gel to check the quality of amplification. To purify the ITS fragments, 20 μl of each PCR reaction was loaded on a 1.6% TAE agarose gel. The agarose blocks containing ITS fragments were excised from the gel with a scalpel under UV light, and applied to the QIAEX gel extraction kit according to the manufacturer's protocol (QIAGEN AG, Basel, Switzerland). ITS fragments were recovered in 20 μl water.

Both strands of purified double-strand DNA were directly sequenced by standard dideoxy chain termination techniques, using primers 5′ end-labelled with digoxigenin (purchased from MWG-Biotech, Germany) and the DIG-TAQ DNA sequencing kit of Boehringer Mannheim GmbH. The following cycling program was performed on a thermal cycler (Perkin Elmer): one cycle of 2 min at 94 °C, linked to 20 cycles of 35 sec at 94 °C, 35 sec at 45 °C and 1 min at 72 °C, followed by 15 cycles of 45 sec at 94 °C and 1 min 30 sec at 72 °C, and finally cooled to 4 °C.

DNA sequencing ladders were separated through a 6% acrylamide – 8M urea gels and transferred directly onto a moving nylon membrane using the GATC-1500 Direct Blotting Electrophoresis DNA Sequencer (MWG-Biotech, Germany). The DNA fragments were cross-linked on the membrane by exposing to UV light for 2 min using the GATC-link (MWG-Biotech). The DNA bands were then detected by anti-digoxigenin using DIG Nucleic Acid Detection Kit of Boehringer Mannheim GmbH. For all the sequencing reactions, electrophoresis and detection of the membrane, the protocols recommended by the manufacturers were followed.

Sequence alignment and phylogenetic analysis. The sequence boundaries between the two spacers and the three coding regions (18S, 5.8S, and 25S genes) of nrDNA were determined by comparison with published sequences from *Daucus carota* and *Vicia faba* (Yokota et al. 1989) and with our previous data (Yuan and Küpfer 1995, Yuan et al. 1996). The combined sequences ITS1 and ITS2 were aligned using the program Clustal X (Thompson et al. 1997) and further adjusted manually.

Potentially informative sites, those with each of at least two character states shared by two or more sequences, were used for phylogeny construction. Phylogenetic trees were reconstructed from the data matrix where gaps were coded as missing, using unweighted Fitch parsimony (Fitch 1971) and ACCTRAN optimisation as implemented in PAUP* 4.0b5 (Swofford 1998). To test the effect of different treatments of gaps on phylogeny reconstruction, additional analyses were carried out where the potentially informative gaps, disregarding their size, were converted into binary characters and were added to the sequence data, or, in one round of analysis, the gap was simply coded as a new state.

Branch-and-Bound searches were conducted in order to find all the most parsimonious trees. In order to access the robustness of the inferred phylogeny, a bootstrap analysis was conducted with 1000 replicates of heuristic searches with TBR branch swapping (Felsenstein 1985). To trace character evolution, life forms, chromosome numbers, sexual expression, and other characters were optimised onto the ITS trees using MacClade 3.0 (Maddison and Maddison 1992). Sequence divergence values between species were calculated for the aligned sequences using the PAUP* with Kimura's two-parameter method (Kimura 1980).

Results

No evidence for multiple repeat types was found. Each PCR product was resolved as a single band on the 1.0% agarose gel. All the sequences were deposited in the EMBL Nucleotide Database. The multiple alignment of all the ingroups and the outgroups required 258 sites for ITS1 and 230 sites for ITS2. Of the aligned 488 sites of ITS1 and ITS2, 196 sites are constant, 156 sites are variable and uninformative (autapomorphic), and 136 sites are informative. The length, G + C content, and accession number of EMBL Nucleotide Database of each sequence are given in Table 2. Although many polyploids were

Table 2. The accession number of EMBL Database, length and G + C content of the ITS1, ITS2 and ITS1 + ITS2 sequences of *Mercurialis* and the outgroups

Sample	ITS1			ITS2			ITS1 + ITS2	
	Accession	Length (bp)	G + C (%)	Accession	Length (bp)	G + C (%)	Length (bp)	G + C (%)
*M. ambigua*62B	AJ488858	230	52.2	AJ488835	213	56.3	443	54.2
*M. ambigua*80B	AJ488859	230	52.6	AJ488836	213	55.4	443	53.9
*M. annua*16M	AJ488860	223	50.7	AJ488837	214	57.9	437	54.2
*M. annua*16F	AJ488861	224	50.4	AJ488838	214	57.5	438	53.8
*M. corsica*66F	AJ488862	225	52.9	AJ488839	216	56.9	441	54.9
*M. elliptica*42M	AJ488863	225	53.8	AJ488840	216	57.9	441	55.8
M. elliptica(aff.) 220M	AJ488865	228	52.6	AJ488842	217	57.2	445	54.9
*M. huetii*16F	AJ488864	229	51.6	AJ488841	215	55.8	444	53.6
*M. leiocarpa*16B	AJ488868	242	44.2	AJ488845	214	51.4	456	47.6
*M. ovata*32M	AJ488869	246	43.5	AJ488846	218	51.4	444	47.2
*M. ovata*16M	AJ488870	244	43.4	AJ488847	216	51.8	460	47.4
*M. perennis*48F	AJ488871	243	42.8	AJ488848	216	51.8	459	47.0
*M. perennis*64M	AJ488872	239	43.5	AJ488849	210	52.4	449	47.6
*M. perennis*64F	AJ488873	241	43.6	AJ488850	210	51.9	451	47.4
*M. perennis*80F	AJ488874	239	43.5	AJ488851	217	50.6	456	46.9
*M. perennis*80M	AJ488875	241	42.7	AJ488852	217	51.6	458	46.9
*M. perennis*96F	AJ488876	243	44.0	AJ488853	215	51.2	458	47.4
*M. reverchonii*26M	AJ488866	229	52.4	AJ488843	215	57.6	444	55.0
*M. tomentosa*26F	AJ488867	226	52.2	AJ488844	215	57.2	441	54.7
Acalypha hispida	AJ488877	228	46.5	AJ488854	194	46.9	422	46.7
Ricinus communis	AJ488878	246	60.6	AJ488855	213	59.2	459	59.9
M. elliptica × *corsica*	AJ488879	225	53.4	AJ488856	216	57.4	441	55.4
M. elliptica × *reverchonii*	AJ488880	229	52.4	AJ488857	216	57.9	445	55.4

included in the present investigation, no clear polymorphic site was observed, except for a few cases of ambiguous sites and the additive polymorphic sites revealed in the putative hybrids (see details below). The length of ITS1 in the ingroups ranged from 223 (*M. annua*) to 246 (*M. ovata*) bps and ITS2 from 210 (*M. perennis*) to 218 (*M. ovata*) bps. The G + C content of the ingroups varied from 42.7% (*M. perennis*) to 53.8% (*M. elliptica*) in ITS1, from 50.6% (*M. perennis*) to 57.9% (*M. annua* and *M. elliptica*) in ITS2, and from 46.9% (*M. perennis*) to 55.8% (*M. elliptica*) in combined ITS1 and ITS2. For each analysed ingroup, the G + C content is obviously higher in ITS2 than in ITS1. Kimura two-parameter sequence divergence distance between pairs of species ranged from 0.0138 (*M. corsica* vs. *M. tomemtosa*) to 0.2394 (*M. ambigua*80B vs. *M. perennis*80M) among the ingroup species in the combined data of ITS1 and ITS2 (distance matrix not shown). No significant variation of sequence was found between the male and female individuals of the species sampled.

Phylogenetic analysis of the ITS sequences employing Fitch parsimony and with gaps coded as missing generated 45 equally maximum parsimonious trees of 460 steps with a consistency index (CI) of 0.85 including autapomorphies (CI = 0.76 excluding autapomorphies) and retention index (RI) of 0.90. The strict consensus of these 45 trees is shown in

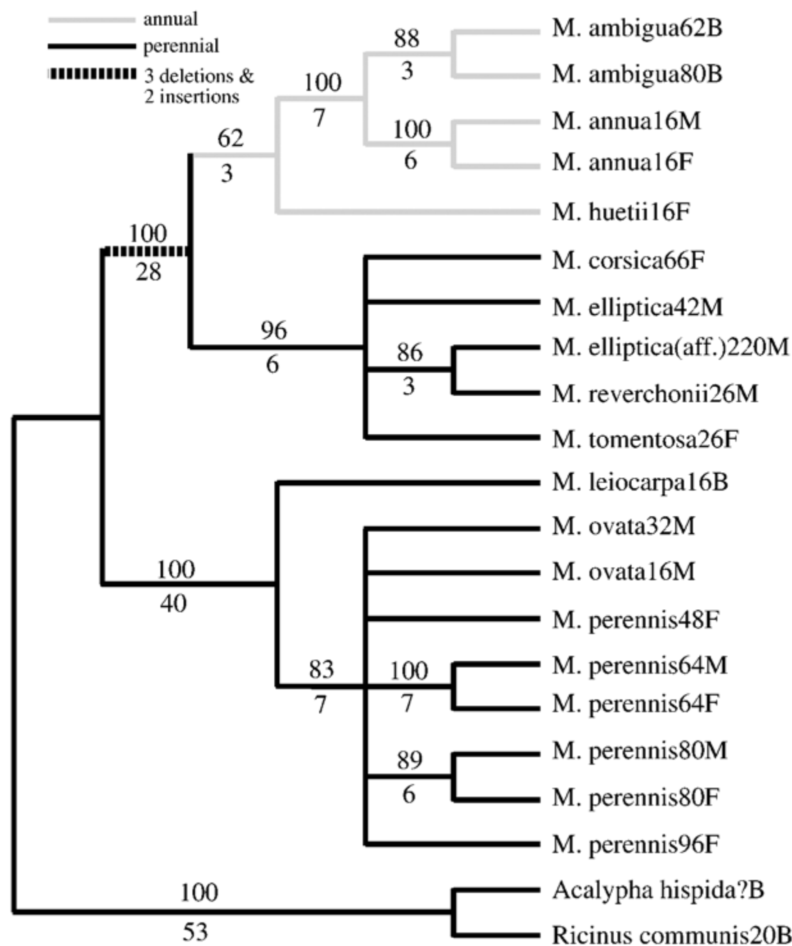

Fig. 1. The strict consensus tree of the 45 equally most parsimonious trees generated from parsimonious analysis on the data matrix where gaps were coded as missing. Tree length = 460, CI = 0.85 including autopomorphies (CI = 0.76 excluding autopomorphies), RI = 0.90. The numbers above the internal branches represent the bootstrap values from 1000 replicates of heuristic searches. The numbers under the internal branches are the numbers of character state changes forming the corresponded consensus branches. Species names are followed by their chromosome numbers and sexual expressions as indicated in Table 1. Life forms (annual versus perennial) and the 5 indel events (represents 3 deletions and 2 insertions) are mapped onto corresponding branches of the tree

Fig. 1. The genus *Mercurialis* was resolved with two principal infrageneric clades. One clade consists of the rhizomatous species *M. perennis*, *M. ovata* and *M. leiocarpa*, which favour shady woods environments and distributed mostly in central to southeastern Europe and eastern Asia. The other clade consists of the rest species of the genus mainly distributed in the western Mediterranean area and ecologically they favour shady, sunny, rocky, or even disturbed grounds (annuals). These two clades were supported by high bootstrap values (both at 100%) and also correspond to 5 indel characters (1–18 bp). Furthermore, G + C contents of these two clades are also obviously different between these two clades: the species of the *M. perennis* clade have lower G + C contents than that of the other (Table 2). Within the western Mediterranean clade, two groups were recognised: the annual clade including *M. annua*, *M. ambigua*, and *M. huetii*, and the perennial group including *M. corsica*, *M. elliptica*, *M. reverchonii* and *M. tomentosa*. In analyses where gaps were treated as extra binary characters or as additional character state of the sites, similar trees were found (not shown) as in the analysis where gaps were coded as missing. Despite the difference in tree length, the principal topology of the trees was concordant with the consensus tree shown in Fig. 1. When characters were optimised onto the ITS tree, the annual life form seems to be a synapomorphic character state derived from perennial (Fig. 1). When chromosome numbers were optimised onto the ITS tree, $x = 8$ is revealed as plesimorphic state

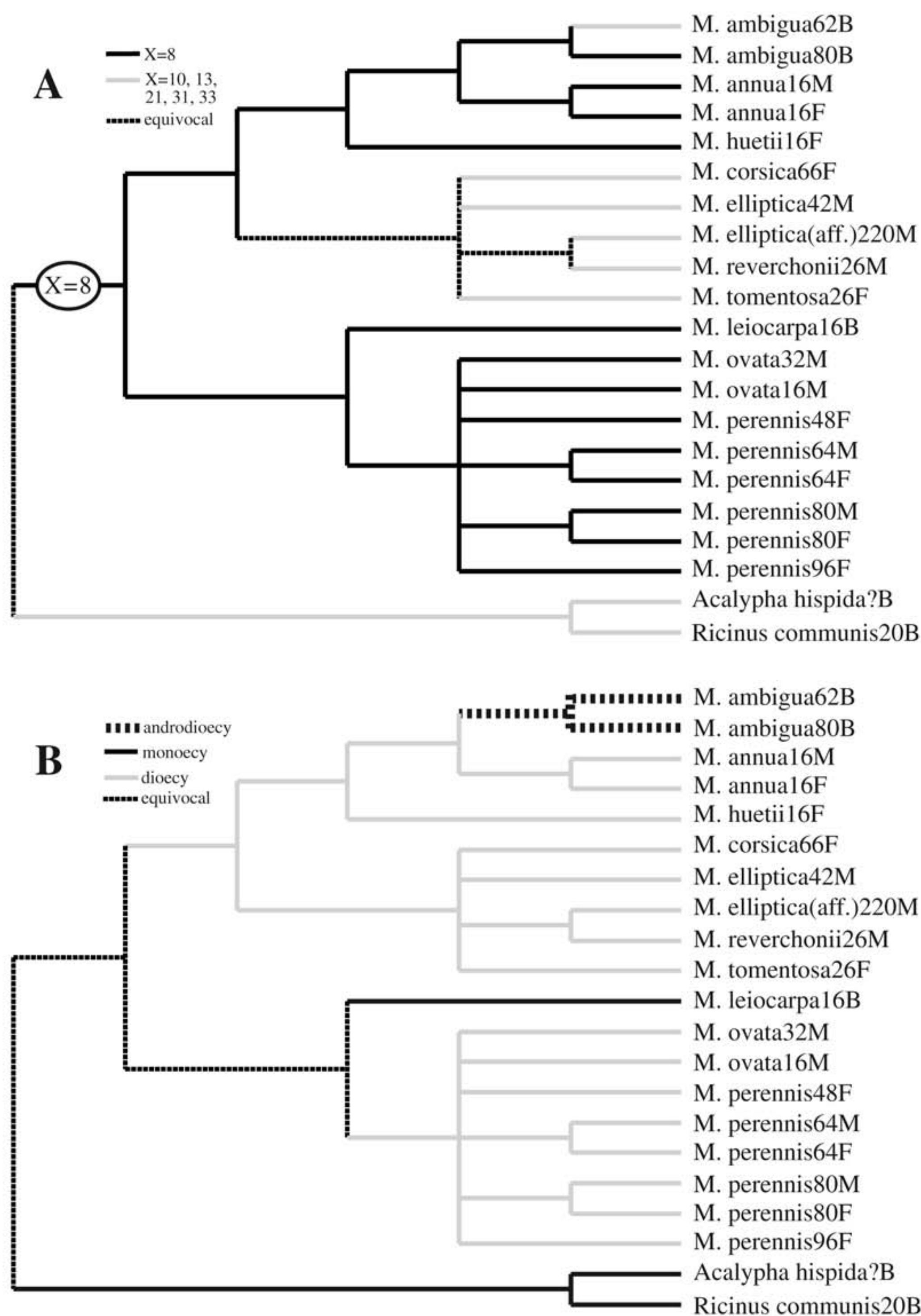

Fig. 2. Character evolution traced with ITS phylogeny. **A** Chromosome numbers optimised onto the concensus ITS tree showing that the basic number $x = 8$ as plesimorphic condition for the genus. **B** Sexual expressions optimised onto the concensus ITS tree showing that the plesimorphic state of the genus as equivocal of dioecy or monoecy, and the androdioecious condition in *M. ambigua* as derived from dioecious. Species names are labelled in the same way as in Fig. 1

for the whole genus (Fig. 2A). The extensive basic number variations are limited to the perennial lineage of the western Mediterranean clade, where interspecific hybridisation is common (see also below). While the monoecy and dioecy remain equivocal as ancestral states for the whole genus, the monoecy/androdioecy of *M. ambigua* is apomorphic, derived from dioecy. If dioecy can be considered as ancestral for the whole genus, monoecy should have been independently evolved twice (Fig. 2B).

To verify putative hybridisation, 632 nucleotide sites (20 of 18S gene, 225 of ITS1, 162 of 5.8S gene, 216 of ITS2 and 9 of 25S gene) from *M. corsica*, *M. elliptica* and their putative hybrid *M. corsica* × *M. elliptica* were compared. Ten divergent sites (four in ITS1 and six in ITS2) were found between *M. corsica* and *M. elliptica*, which exactly corresponded to ten heterozygous sites in the hybrid *M. corsica* × *M. elliptica*. The same sequence of *M. elliptica* was also compared with the 635 nucleotide sequence of *M. reverchonii* and their putative hybrid *M. elliptica* × *M. reverchonii*. 15 nucleotide substitutions (4 in ITS1 and 11 in ITS2) and two indels (insertions/deletions) involving 5 nucleotide sites (one 4 bp indel in ITS1 and one 1 bp indel in ITS2) were found between *M. elliptica* and *M. reverchonii*. All of these 20 divergent sites exactly correspond to the polymorphic sites in the hybrid *M. elliptica* × *M. reverchonii* (see Fig. 3). Agarose gel electrophoresis could not resolve the length polymorphism due to indels and showed only a single band of PCR product. When the PCR products of the putative hybrids were sequenced, gels gave double bands and became unreadable beyond the end of the indel sites. By sequencing complementary strands using both external and internal primers, we were able to confirm the sequences to be exactly additive of the two sequences of the putative parents. These sequence additivity in the ITS regions clearly conforms the interspecific hybridisation in the genus *Mercurialis*.

Discussion

1. Infrageneric phylogeny

ITS phylogeny (Fig. 1) strongly suggest the monophyly of the genus *Mercurialis* with two main infrageneric clades: the *M. perennis* clade including the three rhizomatous species (*M. perennis*, *M. ovata* and *M. leiocarpa*) and the western Mediterranean clade including the remaining seven species of the genus. The divergence of these two infrageneric clades was also supported by different G + C contents of their ITS sequences, and was congruent not only with morphology but also with geographical distribution of the species. The *M. perennis* complex is a typical Eurasian group. It consists of three stoloniferous perennial species: *M. leiocarpa* – the only species of the genus occurring in eastern Asia, *M. ovata* occurring in central and southeastern Europe (mainly in the eastern Mediterranean countries such as Greece and Turkey), and *M. perennis* is widely distributed in most of Europe. The western Mediterranean clade includes seven species: *M. annua* is widespread in the western Mediterranean countries and other parts of Europe, while the others, *M. ambigua*, *M. huetii*, *M. corsica*, *M. reverchonii* and *M. tomentosa* are restricted to the islands and the coastal countries of the western Mediterranean region. Therefore, such a divergence may represent an early split of the genus into two lineages and subsequent development of each lineage into a group of closely related species toward different directions under different evolutionary constraints. The concordance of the ITS tree with the morphological and phytogeographical information suggests that the ITS tree is an accurate estimate of the true phylogeny of the genus *Mercurialis*, at least at the level of main subdivisions.

Within the clade of the *M. perennis* complex, the diploid cytotype of *M. leiocarpa* from China was revealed to be basal. The polyploids of *M. perennis* and *M. ovata* are derived types. However, the relationships among the different cytotypes of *M. perennis* and *M. ovata* cannot be fully resolved by ITS phylogeny (remained

```
corsica66         aacctgcggaaggatcattgTCGAAACCTGCACCTCGCAGAATGACCCGC
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ..................................................
reverchoni26      ..................................................

corsica66         GAACGTGTTTAAAAGATAGTTGGGGGAATGTGTGGCTCCGCGCCCCTCAT
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ..................................................
reverchoni26      ..................................................

corsica66         TCTCCTGACGCAGATGGGAGGGACTTCGGGCGAAT----GCTCATCTCTG
cor_ell54         ................................R..----....R....Y.
elliptica42       ................................G..----....G....C.
ell_rev34         ................................R..TCCC.Y..G....Y.
reverchoni26      ...................................TCCC.T..G......

corsica66         CCGTCTGCTCAACAACCAACCCCGGCGCAGTACGCCAAGGAATAGAAAAT
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ...................................S..............
reverchoni26      ...................................S..............

corsica66         GAAAAGGGCGTGCTTTTCTTTCGGAACGCATTGCCTTATTTCAAGAATCa
cor_ell54         ..........................Y.......................
elliptica42       ..........................T.......................
ell_rev34         ..........................T.......................
reverchoni26      ..........................T.......................

corsica66         aaatgactctcggcaacggatatctcggctctcgcatcgatgaagaacgc
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ..................................................
reverchoni26      ..................................................

corsica66         agcaaaatgcgatacttggtgtgaattgcagaatcccgtgaatcatcgag
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ..................................................
reverchoni26      ..................................................

corsica66         tttttgaacgcaagttgtgcccgaacgtttcggccaaggcacgcctgcct
cor_ell54         ..................................................
elliptica42       ..................................................
ell_rev34         ..................................................
reverchoni26      ..................................................
```

Fig. 3. The aligned sequences of ITS region region (ITS1 + 5.8s gene + ITS2) of *M. corsica*, *M. elliptica*, *M. reverchonii*, and their putative hybrids, showing the sequence additivities in the hybrids. corsica66 = *Mercurialis corsica* (2n = 66), elliptica42 = *M. elliptica* (2n = 42), reverchoni26 = *M. reverchonii* (2n = 26), and the putative hybrids, cor_ell54 = *M. corsica* × *M. elliptica* (2n = 54), ell_rev34 = *M. elliptica* × *M. reverchonii* (2n = 34). Lowercase = gene sequence, uppercase = spacer sequence

```
corsica66        gggtgtcacgcATACGTCGCCCCATCCTCTCGACACCTCGGGAGTCGTGG
cor_ell54        ................WY.....R.....................S.Y..
elliptica42      ................AT.....G.....................G.C..
ell_rev34        ................WY.....R.....................S.Y..
reverchoni26     ..................................................

corsica66        GAGCAGAAGTTGGCCTCCTGTGTGCCTTGCGCATGTGGTTGGCCCAAAAT
cor_ell54        ..................Y...............................
elliptica42      ..................C...............................
ell_rev34        ..................Y.Y.............................
reverchoni26     ....................C.............................

corsica66        TTATGTTCGCGGCAAAATGATAGCCATGACGATCGGTGGTTG TAAAGACC
cor_ell54        ..................................................
elliptica42      ..................................................
ell_rev34        .............M....................................
reverchoni26     .............C....................................

corsica66        CTCTGAAAAAGTCGTGCGCTGTTCTTAGCCGTCGGGACATTCCTTGACCC
cor_ell54        ..................................................
elliptica42      ..................................................
ell_rev34        .......W...............................W..........
reverchoni26     .......T...............................A..........

corsica66        TAGGGCGTCCTCAGGGCCGCTCCGACCgcgacccca
cor_ell54        ....................................
elliptica42      ....................................
ell_rev34        ................S...................
reverchoni26     ................G...................
```

Fig. 3 (continued)

as a polytomy), which suggest the polyploids arose within a short period of time, probably recently. This situation indicates that, on the one hand, these cytotypes are indeed closely related to each other, and on the another hand, these cytotypes, although stabilised, are probably not completely isolated from each other and occasional gene flow may exist among them through hybridisation between different cytotypes. Furthermore, cytotypes of *M. perennis* and *M. ovata* are not resolved as monophyletic clades corresponding to the species delimitations, suggesting rather close relationship or incomplete differentiation between them.

Within the clade of western Mediterranean species, two lineages can be recognised by ITS sequences: the annual one including *M. annua*, *M. ambigua*, and *M. huetii*, and the perennial one including *M. corsica*, *M. elliptica*, *M. reverchonii* and *M. tomentosa*. Since perennial life form is plesiomorphic in this genus, the annual form is then a good apomorphic character of the group. Within the annual lineage, *M. huetii* occurs as a basal species, while *M. ambigua* and *M. annua* are closely related. The relationships among the species of the perennial lineage are not unambiguously resolved, which suggest a recent extensive differentiation or frequent interspecific hybridisation where different species contact.

2. Chromosomal evolution

Karyological studies have revealed extensive chromosomal variation in the genus *Mercurialis* (Krähenbühl 1984, Krähenbühl and Küpfer 1995, Durand and Durand 1992). Intraspecific polyploids are very common in the genus, and most species (or cytotypes) have

the basic number $x = 8$. The extreme example of chromosome variation is represented by *M. perennis*, for which about 43 different cytotypes have been described. The extreme wide range of chromosome numbers include polyploidy, aneuploidy and B chromosomes and likely arose from hybridisation of different cytotypes (Krähenbühl 1984, Krähenbühl and Küpfer 1995). Cytogeographical considerations suggested $2n = 16$ to be the ancestral form of the complex (as well as the genus). From this form, two vicariants, the oriental type of *M. leiocarpa* and occidental type of diploid *M. ovata* were derived. From the occidental type, in turn, the other polyploid cytotypes of *M. ovata* and *M. perennis* were formed (Krähenbühl and Küpfer 1995). The variation of basic chromosome numbers is limited within the perennial lineage of the western Mediterranean clade, where ITS phylogeny is poorly resolved, which suggests that the evolution of different basic numbers were relatively recent events, and/or frequent hybridisations were involved. Despite the poor resolution of the ITS gene tree among the closely related cytotypes, optimisation of the chromosome numbers on the ITS trees does suggest that $x = 8$ is the plesiomorphic state for the whole genus and also for the clade of the *M. perennis* complex (Fig. 2A). This conclusion confirmed the karyological suggestions.

3. *Evolution of breeding systems*

While most species of *Mercurialis* are dioecious, different cytotypes of *M. ambigua* are monoecious or androdioecious, i.e. both male individuals and cosexual monoecious individuals exist in a population. Theoretical studies have attempted to explain androdioecy as having evolved from hermaphroditism (Charlesworth 1984). Rieseberg et al. (1992) initially concluded that the androdioecy in *Datisca* had been evolved from dioecy based on a phylogenetic reconstruction of Datiscaceae using DNA restriction data. Recent further investigations employing both chloroplast and nuclear DNA sequence data and extended outgroups showed that the family Datiscaceae were not monophyletic and whether dioecy or monoecy was ancestral to androdioecy was equivocal (Swensen et al. 1994, 1998). Pannell (1997) believed that dioecy was the ancestral condition in *Mercurialis* and thus considered monoecy and androdioecy as a derived as a state. His conclusion was based on the fact that most species of the genus are dioecious, without considering the phylogenetic relationships among the species and the Asiatic species *M. leiocarpa*. When the breeding systems are optimised onto the ITS phylogeny, the monoecy/androdioecy of *M. ambigua* is confirmed as derived from dioecy, while the ancestral condition of the whole genus remains equivocal for dioecy or monoecy (Fig. 2B). The sexual expression of *M. leiocarpa* is somehow critical in assessing the plesimorphic state of the whole genus. In some literature this species is described as dioecious (e.g. IB-CAS 1994), while in some others as monoecious (e.g. Qiu 1996). Our study of herbarium specimens have revealed male, female and co-sexual individuals. Thus this species is probably polygamous, a 'combination' of both dioecy and monoecy. No explicit statistical studies have been done on the relative proportions of different sexual expressions of this species in natural populations. If its dioecy can be confirmed, then the ancestral plesiomorphic state of the whole genus becomes unequivocally dioecy. As we saw more monoecious herbarium specimens and the plants we used for DNA extraction were monoecious, we presented the species as monoecious in Fig. 2B. Besides, unlike *M. ambigua* where androdioecy is linked to higher chromosome numbers (ploidy levels), the samples of *M. leiocarpa* we used for DNA analyses were diploid ($2n = 16$) and monoecious. An extensive study on the sexual expression of *M. leiocarpa* is needed. The assessment of the ancestral state of sexual expression of the genus *Mercurialis* also clearly depends on the choice of outgroups. So far, the sister groups of *Mecurialis* have not been confirmed in a phylogenetic way. A study on the entire tribe Acalypheae should resolve this problem.

Nevertheless, it can be confirmed that the androdioecy in *M. ambigua* was derived from dioecy (Fig. 2B).

4. Hybridisation

Interspecific hybridisation in *Mercurialis* had been noticed long ago (Ascherson and Graebner 1913, Pax and Hoffmann 1914), for example, for the origin of *M.* × *paxii* (*M. perennis* × *M. ovata*). Hybridisation between *M. elliptica* and *M. tomentosa* was also suggested (Tutin et al. 1968). Our observations on transplanted samples in the botanical garden suggested that hybrids could arise spontaneously when some species come into contact with each other. At least *M. elliptica* can easily hybridise with *M. corsica* or *M. reverchonii*. The additivity of the ribosomal DNA sequences confirmed the hybridization (Fig. 3). The samples we analysed were probably the F1 individuals, as the rDNAs have not yet been homogenised by concerted evolution (Hillis et al. 1991). So far we do not have clear conclusions on the fertilities of the hybrid individuals, and thus we do not know yet the fortune of the hybrid individuals within natural populations. Considering the perennial life form of the hybrids, the occurrence of interspecific hybrids can cause confusions in taxonomic determination and chromosome number counting. Interestingly, the widest basic chromosome number variation reported so far is limited to the clade where we observed frequent hybridisation. These results suggested that the spatial and/or ecological isolations are important isolation mechanisms among many species of *Mercurialis*.

We are indebted to Dr. Xue-Jun Ge for his help in studying the herbarium specimens of *Mercurialis leiocarpa*. Thanks to two anonymous reviewers for their suggestions on the manuscript. This investigation was financially supported by Swiss National Scientific Foundation granted to Philippe Küpfer (3100-052885) and a visiting scholarship of the Ministry of Education of the People's Republic of China granted to Yong-Ming Yuan.

References

Ascherson P., Graebner P. (1913) *Mercurialis*. Synopsis der Mitteleuropäischen Flora. 7: 403–411.

Baldwin B. G. (1992) Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Molec. Phyl. Evol. 1: 3–16.

Baldwin B. G. (1993) Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. Amer. J. Bot. 80: 222–238.

Baldwin B. G., Sanderson M. J., Porter J. M., Wojciechowski M. F., Campbell C. S., Donoghue M. J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on Angiosperm family. Ann. Missouri Bot. Gard. 82: 247–277.

Ballard H. E., Sytsma K. J. (2000) Evolution and biogeography of the woody Hawaiian violets (*Viola*, Violaceae): arctic origins, herbaceous ancestry and bird dispersal. Evolution 54: 1521–1532.

Charlesworth D. (1984) Androdioecy and the evolution of dioecy. Biol. J. Linn. Soc. 22: 333–348.

Doyle J. J., Doyle J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull. 19: 11–15.

Durand R., Durand B. (1992) Dioécie, monoécie, polyploïdie et spéciation chez les Mercuriales annuelles. Bull. Soc. Bot. France, Lettres Botanique 139 (4/5): 377–390.

Felsenstein J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Fitch W. M. (1971) Toward defining the course of evolution: minimum change for a specific tree topology. Syst. Zool. 20: 406–416.

Francisco-Ortega J., Park S.-J., Santos-Guerra A., Benabid A., Jansen R. K. (2001) Origin and evolution of the endemic Macaronesian Inuleae (Asteraceae): evidence from the internal transcribed spacers of nuclear ribosomal DNA. Biol. J. Linn. Soc. 72: 77–97.

Hillis D. M., Moritz C., Porter C. A., Baker R. J. (1991) Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science 251: 308–310.

IB-CAS (Institute of Botany, Chinese Academy of Sciences) (1994) Iconographia Cormophytorium Sinicorum Vol. II, p.603. Science Press, Beijing (in Chinese).

Kimura M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Krähenbühl M. (1984) Le complexe polyploïde et aneuploïde du *Mercurialis perennis* L. dans le canton de Neuchâtel. Bull. Soc. Neuchâtel. Sci. Nat. 107: 39–54.

Krähenbühl M., Küpfer P. (1995) Le genre *Mercurialis* (Euphorbiaceae): cytogéogrphie et évolution du complexe polyploïde des *M. perennis.*, *M. ovata* Sternb. & Hoppe et *M. leiocarpa* Sieb. & Zucc. Candollea 50: 411–430.

Liston A., Rieseberg L. H., Elias T. S. (1990) Functional androdioecy in the flowering plant *Datisca glomerata*. Nature 343: 641–642.

Maddison W. P., Maddison D. R. (1992) MacClade 3.0. Interactive analysis of phylogeny and character evolution. Sinauer, Sunderland, MA.

Panero J. L., Francisco-Ortega J., Jansen R. K., Santos-Guerra A. (1999) Molecular evidence for multiple origins of woodiness and a new world biogeographic connection of the macaronesian Island endemic *Pericallis* (Asteraceae: Senecioneae). Proc. Natl. Acad. Sci. USA. 96: 13886–13891.

Pannell J. (1997) Widespread functional androdioecy in *Mercurialis annua* L. (Euphorbiaceae). Biol. J. Linn. Soc. 61: 96–116.

Pax F., Hoffmann K. (1914) Euphorbiaceae-Acalypheae-Mercurialineae. In: Engler A. (ed.) Das Pflanzenreich 4(147.7): 1–288.

Qiu Huaxing (1996) *Mercurialis*. In: Qiu Huaxing (ed.) Euphorbiaceae. Flora Reipublicae Popularis Sinicae 44(2). Science Press, Beijing (in Chinese), p. 82.

Richards A. J. (1997) Plant breeding systems, 2nd edn. Chapman & Hall, London.

Rieseberg L. H., Hanson M. A., Philbrick C. T. (1992) Androdioecy is derived from dioecy in Datiscaceae: evidence from restriction site mapping of PCR-amplified chloroplast DNA fragments. Syst. Bot. 17: 324–336.

Sang T., Crawford D. J., Stuessy T. F. (1997) Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA 92: 6813–6817.

Schmidt G. J., Schilling E. E. (2000) Phylogeny and biogeography of *Eupatorium* (Asteraceae: Eupatorieae) based on nuclear ITS sequence data. Amer. J. Bot. 87: 716–726.

Swensen S. M., Mullin B. C., Chase M. W. (1994) Phylogenetic affinities of Datiscaceae based on an analysis of nucleotide sequences from the plastid *rbc*L gene. Syst. Bot. 19: 157–168.

Swensen S. M., Luthi J. N., Rieseberg L. H. (1998) Datiscaceae revisited: monophyly and the sequence of breeding system evolution. Syst. Bot. 23: 157–169.

Swofford D. L. (1998) PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4.0. Sinauer, Sunderland, Massachusetts.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882.

Tutin T. G., Heywood V. H., Burges N. A., Moore D. M., Valentine D. H., Walters S. M., Webb D. A. (1968) Flora Europaea vol. 2. Cambridge University Press, Cambridge.

Valedes B., Talavera S., Fernandez-Galiano E. (1987) Flora vascular de Andalucia Occidental vol. 2. Ketres Editora, Bacelona.

Vargas P., Morton C. M., Jury S. L. (1999) Biogeographic patterns in Mediterranean and Macaronesian species of *Saxifraga* (Saxifragaceae) inferred from phylogenetic analyses of ITS sequences. Amer. J. Bot. 86: 724–734.

Webster G. L. (1994) Synopsis of the genera and suprageneric taxa of Euphorbiaceae. Ann. Missouri Bot. Gard. 81: 33–144.

Wendel J. F., Schnabel A., Seelanan T. (1995) Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci., USA 92: 280–284.

White T. J., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M., Gelfand D., Sninsky J., White T. (eds.) PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, pp. 315–322.

Yokota Y., Kawata T., Iida Y., Kato A., Tanifuji S. (1989) Nucleotide sequences of the 5.8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. J. Molec. Evol. 29: 294–301.

Yuan Y.-M., Küpfer P. (1995) Molecular phylogenetics of the subtribe *Gentianinae* (Gentianaceae) inferred from the sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA. Plant Syst. Evol. 196: 207–226.

Yuan Y.-M., Küpfer P., Doyle J. J. (1996) Infrageneric phylogeny of the genus *Gentiana* (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Amer. J. Bot. 83: 641–652.

Address of the authors: Martin Krähenbühl, Yong-Ming Yuan (correspondence) and Philippe Küpfer, Institut de Botanique, Laboratoire de botanique évolutive, Université de Neuchâtel, Emile-Argand 11, CH-2007 Neuchâtel, Suisse. (e-mail: yong-ming.yuan@unine.ch)