The Evolution of Reproductive Systems and Sex-Determining Mechanisms Within *Rumex* (Polygonaceae) Inferred from Nuclear and Chloroplastidial Sequence Data

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The genus *Rumex* includes hermaphroditic, polygamous, gynodioecious, monoecious, and dioecious species, with the dioecious species being represented by different sex-determining mechanisms and sex-chromosome systems. Therefore, this genus represents an exceptional case study to test several hypotheses concerning the evolution of both mating systems and the genetic control of sex determination in plants. Here, we compare nuclear intergenic transcribed spacers and chloroplast intergenic sequences of 31 species of *Rumex*. Our phylogenetic analysis supports a systematic classification of the genus, which differs from that currently accepted. In contrast to the current view, this new phylogeny suggests a common origin for all Eurasian and American dioecious species of *Rumex,* with gynodioecy as an intermediate state on the way to dioecy. Our results support the contention that sex determination based on the balance between the number of X chromosomes and the number of autosomes (X/A balance) has evolved secondarily from male-determining Y mechanisms and that multiple sex-chromosome systems, XX/XY_1Y_2, were derived twice from an XX/XY system. The resulting phylogeny is consistent with a classification of *Rumex* species according to their basic chromosome number, implying that the evolution of *Rumex* species might have followed a process of chromosomal reduction from x = 10 toward x = 7 through intermediate stages (x = 9 and x = 8).

Introduction

Studies of the origin and evolution of dioecy and sex-determining mechanisms are two major topics in evolutionary biology. Among flowering plants the origin of dioecy appears to have resulted from quite recent events (Guttman and Charlesworth 1998) that have independently occurred in about 7% of the genera (Renner and Ricklefs 1995). Two pathways have been found for this to occur. Groups in which dioecy evolved from monoecy consist of monoecious and dioecious species, while groups in which dioecy derived from gynodioecy are composed of gynodioecious and dioecious species (Renner and Won 2001).

Within dioecious plant species, however, there are a few species that exhibit chromosome-mediated sex-determination systems (Charlesworth 2002; Ruiz Rejón 2004). The most common case is the existence of XX/XY chromosomal complements and Y-based sex-determining mechanisms. However, there appear to be other alternatives, such as complex chromosomal systems (i.e., XX/XY_1Y_2 systems) and cases in which the sex specification is mediated by the balance between the number of X chromosomes and the number of autosomes (X/A balance). It is assumed that complex chromosomal systems are derived from a simple XX/XY system by some sort of rearrangement such as unequal translocations (Smith 1969). It is also assumed that the X/A balance mechanisms evolved secondarily from male-determining Y-chromosome mechanisms (Westergaard 1958). Circumstantial evidence supporting this assumption is provided by the fact that the X/Y sex determination is taxonomically far more widely distributed than X/A, especially in groups, such as fishes and plants, with relatively poorly developed sex-chromosome systems (discussed in Charlesworth 1996). However, there is no direct evidence supporting that the X/Y system is indeed older.

One way to determine the ancestral sex-determination system and the number of kinds of evolutionary transitions that resulted from it is to overlay character state information onto a phylogenetic tree. In this paper, we perform phylogenetic analyses on members of the genus *Rumex*, in which different sex-determining mechanisms and sex-chromosomal systems are found (Löve 1957; Smith 1969; Charlesworth 1996). In fact, within the genus *Rumex*, we find both X/Y and X/A mechanisms of sex-determination and species that range from being gynodioecious, monoecious, and polygamous to hermaphroditic; therefore, the genus provides an exceptional opportunity to test hypotheses concerning the number and nature of evolutionary transitions involved in sex determination.

The genus *Rumex* is currently divided into four subgenera (table 1): *Acetosella*, *Acetosa*, *Platypodium*, and *Rumex* (Rechinger 1937, 1964; Löve 1944; Löve and Kapoor 1967; Degraeve 1976). *Acetosella* contains two species, *Rumex acetosella* (which has several subspecies) and *Rumex graminifolius*. These species are dioecious and have a sex-determination mechanism based on the presence of an active Y and a simple chromosome system XX/XY (Löve 1944; Smith 1969). Within the subgenus *Acetosa,* the section *Acetosa* is composed of *Rumex acetosa* and its relatives, which form an homogeneous group of species characterized by similar morphological and karyological characteristics, including an XX/XY_1Y_2 sex-chromosome system plus a sex-determination mechanism based on the X/A balance (Löve 1957; Smith 1969; Degraeve 1976; Wilby and Parker 1988; Ainsworth et al. 1999). However, within the section *Americanae* of the subgenus *Acetosa*, there are two species: *Rumex paucifolius*, which has the XX/XY system, and *Rumex hastatulus*, which has two chromosomal races, one with the XX/XY

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Table 1
List of Analyzed *Rumex* Species

Species	SD	X	Procedence	Accession Numbers
Subgenus *Acetosa*				
Section *Acetosa*				
Rumex acetosa	D (XX/XY_1Y_2)	7	Capileira, Granada (Spain), WM	AJ583840, AJ583853, AJ580774, AJ580790
Rumex papillaris	D (XX/XY_1Y_2)	7	La Benajara, S[a] Baza, Granada (Spain), WM	AJ583841, AJ583854, AJ580775, AJ580791
Rumex tuberosus	D (XX/XY_1Y_2)	7	Jabal, Sinjar (Iraq), VSC; S. Omar, A. L. Kaisi and A. L. Khayat 52591, K	AJ698483, AJ699267, AJ810978, AJ810988
Rumex intermedius	D (XX/XY_1Y_2)	7	Vollubilis (Morocco), WM	AJ583847, AJ583860, AJ580781, AJ580796
Rumex thyrsoides	D (XX/XY_1Y_2)	7	Vollubilis (Morocco), WM	AJ583848, AJ583861, AJ580780, AJ580797
Section *Americanae*				
Rumex hastatulus (NCR)	D (XX/XY_1Y_2)	4	Cumberland County, North Carolina, WM	AJ698484, AJ699268, AJ704987, AJ704988
Rumex hastatulus (TXR)	D (XX/XY)	5	Masan County, Texas, WM	AJ698485, AJ699269, AJ810979, AJ810989
Rumex paucifolius	D (XX/XY)	7	Challis National Forest, Custer County, Idaho, VSC; R. Steele 702, BOISE	AJ889018, AJ889019, AJ890454, AJ890455
Section *Scutati*				
Rumex scutatus	H/P	10	S[a] Mágina, Jaén (Spain), WM	AJ583838, AJ583851, AJ580777, AJ580793
Rumex induratus	H/P	10	Padul, Granada (Spain), WM	AJ583837, AJ583850, AJ580778, AJ580794
Rumex roseus	H/P	10	Tarancón, Cuenca (Spain), WM	AJ812000, AJ811999, AJ844275, AJ844276
Rumex suffruticosus	D (XX/XY)	8	Pto. Navacerrada, Segovia (Spain), WM	AJ583849, AJ583862, AJ580782, AJ580786
Section *Hastati*				
Rumex hastatus	P/G	9	Zhongdian, Yunnan (China), VSC; ACE 294, K	AJ698488, AJ6992672, AF338218*
Rumex maderensis	P/G	10	Real Jardín Botánico de Madrid (Spain), C	AJ810929, AJ810939, AJ810980, AJ810990
Rumex lunaria	P/G	9	Gáldar, Gran Canaria (Spain), WM	AJ583839, AJ583852, AJ580779, AJ580795
Section *Vesicarii*				
Rumex vesicarius	H/P	9	Presa de Ayagures, Gran Canaria (Spain), WM	AJ889247, AJ889248, AJ889016, AJ889017
Rumex cyprius	H/P	9	Khorio, Kalio (Cyprus), VSC; A. Dellon ARI13094, K	AJ810935, AJ810945, AJ810981, AJ810991
Section *Afroacetosa*				
Rumex abyssinicus (SsA)	P	9	Gilo, Imatong Mts. (Sudan), VSC; I. Friis and K. Vollesen 87, K	AJ698487, AJ699271, AJ844278, AJ844279
Rumex sagittatus (SsS)	D	9	Montagu Island, New South Wales (Australia), VSC; P. C. Heyligers 89028, K	AJ698486, AJ699270, AJ889014, AJ889015
Subgenus *Acetosella*				
Rumex acetosella	D (XX/XY)	7	Capileira, Granada (Spain), WM	AJ583842, AJ583855, AJ580776, AJ580792
Rumex graminifolius	D (XX/XY)	7	Tjumen, Jamal Peninsula (Russia), VSC; O. Rebristaya 6315, K	AJ810934, AJ810944, AJ831539, AJ844277
Subgenus *Rumex*				
Rumex patientia	H	10	Real Jardín Botánico de Madrid (Spain), C	AJ810931, AJ810941, AJ810984, AJ810994
Rumex pulcher	H	10	Guadarrama, Madrid (Spain), WM	AJ810930, AJ810940, AJ810983, AJ810993
Rumex conglomeratus	H	10	Atarfe, Granada (Spain), WM	AJ583843, AJ583856, AJ580785, AJ580789
Rumex crispus	H	10	Atarfe, Granada (Spain), WM	AJ583844, AJ583857, AJ580784, AJ580788
Rumex cristatus	H	10	Cercedilla, Madrid (Spain), WM	AJ704864, AJ704865, AJ844272, AJ844273
Rumex obtusifolius	H	10	Guadarrama, Madrid (Spain), WM	AJ810927, AJ810937, AJ810985, AJ810995
Rumex sanguineus	H	10	Real Jardín Botánico de Madrid (Spain), C	AJ810928, AJ810938, AJ810987, AJ810997
Rumex aquitanicus	H	10	Real Jardín Botánico de Madrid (Spain), C	AJ810932, AJ810942, AJ810986, AJ810996
Rumex japonicus	H	10	Nigel Huneyman, personal collection, C	AJ810936, AJ810946, AF338220*
Rumex giganteus	M	10	Pohakoloa, Hawaiian Islands, Hawaiian Plant DNA Library (HPDL), University of Hawaii at Manoa	AJ698482, AJ699266, AJ810982, AJ810992
Subgenus *Platypodium*				
Rumex bucephalophorus	H	8	Padul, Granada (Spain), WM	AJ583846, AJ583859, AJ580783, AJ580787
Fallopia convolvulus	H	10	Capileira, Granada (Spain), VSC; J. Molero 10719, GDA	AJ583845, AJ583858, AF040064*

NOTE.—Species: NCR, North Carolina Race; TXR, Texas Race; SsA, subsection *Abyssinici*; SsS, subsection *Sagittati*. Sex-determination systems (SD): H, hermaphrodite; P, polygamous; G, gynodioecious; M, monoecious; D, dioecious. Basic chromosome number (X). Procedence: WM, wild material; C, cultivar; VSC, voucher specimen code. Herbarium codes: BOISE, Rocky Mountain Research Station Herbarium; DUKE, Duke University Herbarium; GDA, University of Granada Herbarium, Spain; K, Royal Botanic Gardens, Kew, United Kingdom. Accession numbers: intron, spacer, ITS1 and ITS2 except for * (intron, spacer, ITS1-2).

(called the "Texas race") and the other with the XX/XY_1Y_2 (called the "North Carolina race") (Smith 1969). Also, the second race has an X/A-based sex-determination mechanism, while the XX/XY race has a Y-based one. Furthermore, the subgenus *Acetosa* contains four additional sections: *Scutati, Vesicarii, Hastati,* and *Afroacetosa*. The first two are composed of hermaphroditic and polygamous species. Strikingly, *Scutati* has a dioecious species, *Rumex suffruticosus*, for which no chromosomal data are available (López González 1990). The sections *Hastati* and *Afroacetosa* are composed of polygamous and gynodioecious species as well as a dioecious one, *Rumex sagittatus,* which lacks differentiated sex chromosomes (Degraeve 1976). Meanwhile, the third subgenus, *Platypodium*, has one species (and several subspecies), *Rumex bucephalophorus,* which is hermaphroditic. Finally, the

subgenus *Rumex* is composed of hermaphroditic species, although endemic Hawaiian species such as *Rumex giganteus* have evolved towards monoecy (Wagner, Herbst, and Sohmer 1999). If this classification reflects the phylogeny of the genus, it implies that dioecy has appeared several times over the evolution of *Rumex* species directly from a hermaphroditic ancestor. According to this classification there involves that there has been no evolutionary constraint on the evolution of sexual systems and that forward and reverse evolution occur with equal probability. Here, we test whether or not these assumptions are correct by means of a phylogenetic analysis of the genus *Rumex* based on one nuclear and one chloroplastidial marker. Specifically, we seek to address (1) whether dioecy has appeared once or several times in *Rumex*, (2) whether the Y-based sex-determination mechanism precedes to the X/A mechanism, (3) whether the multiple sex-chromosome system is derived from an XX/XY system, and (4) whether a different infrageneric classification can be proposed for *Rumex* species consistent with their mating system and with their karyotype evolution.

Materials and Methods

Table 1 lists the names of the 31 *Rumex* species studied in this paper, according to the subgenera to which they are currently ascribed and according to specifics such as reproductive system, sex-determining mechanism, chromosome number, or sex-chromosome system. The species *Fallopia convolvulus* (Polygonaceae) was used as the out-group. The DNA was extracted from fresh leaf samples by the guanidine-detergent lysing method using the Plant DNAzol kit (Invitrogen, Carlsbad, Calif.), following the manufacturer's recommendations. The material for several *Rumex* species was taken from specimens donated by different herbariums (see table 1), and in these cases the DNA was obtained by the method of J. J. Doyle and J. L. Doyle (1987).

We analyzed two DNA regions in this study. From the nuclear genome, we analyzed the intergenic transcribed spacers (ITS) between the 18S and the 28S ribosomal genes. From the chloroplast genome, we have analyzed the intron sequence of the *trn*L gene and the intergenic spacer between this gene and the *trn*F gene. The complete ITS region, including the ITS1, the 5.8S gene, and the ITS2, was amplified by polymerase chain reaction (PCR) using the ITS1 and ITS4 primers (White et al. 1990) for a few species, and afterwards we designed the *Rumex* specific primers, ITS1F (5′-AAGGTTTCCGTAGGTGAACC-3′), ITS1R (5′-AGATATCCGTTGCCGAGAGT-3′), ITS2F (5′-AGTCTTTGAACGCAAGTTGC-3′), and ITS2R (5′-CCTCCGCTTATTGATATGCT-3′) for the rest of species. The *trn*L intron and the *trn*L-*trn*F intergenic spacer regions of the chloroplast genome were amplified by using the primer pair trnL-c/trnL-f (Taberlet et al. 1991). This primer pair amplifies the complete region between the first exon of the *trn*L gene and the *trn*F gene and includes the *trn*L intron, the second exon of *trn*L, and the spacer between this gene and the *trn*F gene. PCR amplifications were made in 50-μl reactions containing 10 ng of purified DNA, 2 mM of deoxynucleoside triphosphate, 2 mM of each primer, and 1.25 U of *Taq* polymerase in 10 mM Tris-HCl at pH 8.3, 5 mM KCl, and 2 mM MgCl reaction buffer. For the ITS, the PCR reaction was displayed in 40 thermal cycles consisting of 1 min at 94°, 1 min at 55°, and 1 min at 72°, while for the chloroplast region, 33 thermal cycles were displayed consisting of 1 min at 93°, 1 min at 50°, and 1 min at 72°.

The PCR products were ligated to the cloning plasmid pGEM-Teasy (Promega, Madison, Wisc.) and cloned in competent *Escherichia coli* JM109 cells (Promega) following the manufacturer's recommendations. From each species, several clones of every marker were sequenced by the dideoxy sequencing method using the automatic ABI-Prism 377 sequencer system (Applied Biosystems, Foster City, Calif.). The European Molecular Biology Laboratory (EMBL) accession numbers for all the sequences analyzed in this study are indicated in table 1. The ITS sequences of *F. convolvulus, Rumex hastatus*, and *Rumex japonicus* were taken from the EMBL database under the accession numbers AF040064, AF338218, and AF338220, respectively.

For sequence analysis, multiple alignments were performed using ClustalX (Thompson et al. 1997) followed by manual adjustments. Gene sequences were excluded from the alignments. Phylogenetic relationships among taxa were estimated using three different methods: maximum parsimony (MP), maximum likelihood (ML), and neighbor-joining (NJ) methods. MP and ML were implemented by the PAUP* v4b10 program (Swofford 1998) and the trees were displayed with Treeview32 (Page 1996), while NJ was implemented by the MEGA v.2.1 program (Kumar et al. 2001). Gaps were treated as missing data. For MP, heuristic searches were run with 1,000 random taxon-addition replicates using the tree bisection-reconnection algorithm and the Multrees option. For selection of the DNA substitution model for ML and NJ, the aligned sequences were subjected to analysis using Modeltest v.3.6 (Posada and Crandall 1998), which performs a hierarchical test of likelihood fits under 56 different models of DNA substitution. Bootstrap support values were calculated on 1,000 replicates in PAUP* v4b10 (Swofford 1998). Congruence between the nuclear and chloroplast data sets was analyzed with PAUP* v4b10 (Swofford 1998) by conducting a partition-homogeneity test (Incongruence Length Difference Test of Farris et al. 1995) after 1,000 random replicates.

Results

The complete ITS region (ITS1 plus ITS2) varied from 317 to 416 bp in the *Rumex* species. The alignment data set contained 468 characters, 207 of which were informative for parsimony analysis. Alignment required several gaps ranging from 11 to 64 bp in length. Curiously, large deletion-insertion regions were phylogenetically informative (fig. 1). We did not include the indels in the parsimony analysis, but there was phylogenetic clustering of indels with certain groups of sex-determination systems. For example, assuming the ITS1 length of *F. convolvulus* as ancestral, we found a large deletion of 53–63 bp within the ITS1 of all the Eurasian and American dioecious species of *Rumex* (fig. 1). Furthermore, the species having

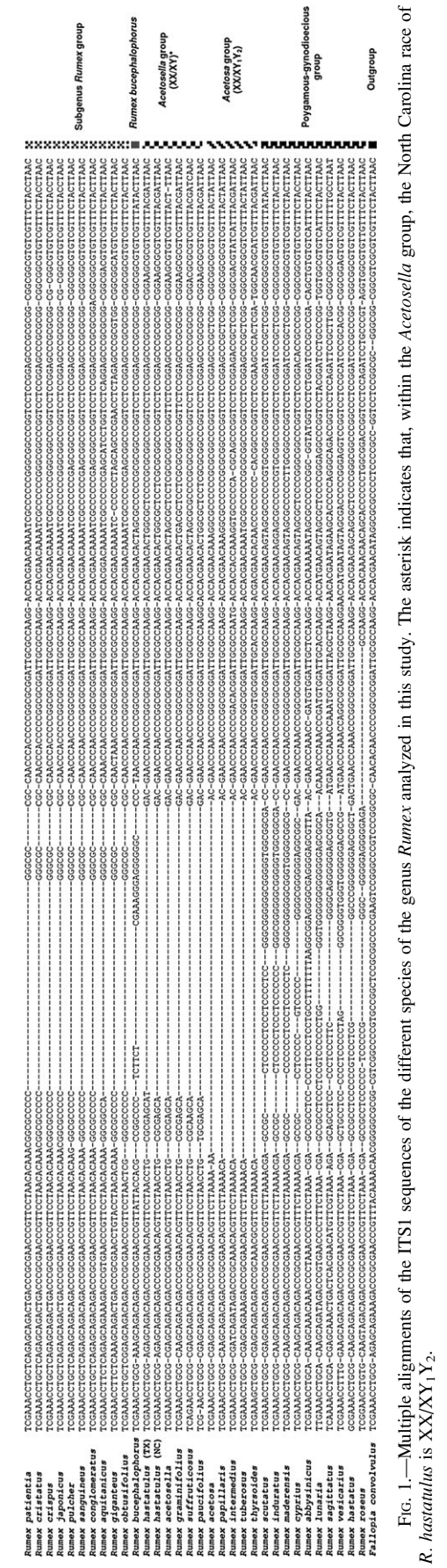


FIG. 1.—Multiple alignments of the ITS1 sequences of the different species of the genus *Rumex* analyzed in this study. The asterisk indicates that, within the *Acetosella* group, the North Carolina race of *R. hastatulus* is XX/XY_1Y_2.

an XX/XY sex-chromosome–determination system (subgenera *Acetosa* and *Acetosella*) had the deletion of 53 bp, while the XX/XY_1Y_2 species (subgenus *Acetosa*) had the deletion of 63 bp. Moreover, shorter deletions in this region distinguished two groups of species—on one hand, the rest of the nondioecious species of *Acetosa* and the African dioecious *R. sagittatus* and, on the other hand, the species of the subgenus *Rumex* (fig. 1).

NJ (Tamura-Nei gamma distance) and ML (HKY + G model of DNA substitution) trees revealed the same topology (fig. 2). The trees reflect three main clades. One clade is composed of the dioecious Eurasian and American species (those of subgenera *Acetosa* and *Acetosella*) and can be divided into two subclades, one composed of the dioecious species of the section *Acetosa* (subgenus *Acetosa*) having the XX/XY_1Y_2 sex-determination chromosomal system and the other composed of the species of the subgenus *Acetosella*, the species of the section *Americanae* of the subgenus *Acetosa* (*R. paucifolius* and *R. hastatulus*), and *R. suffruticosus* (section *Scutati* of *Acetosa*). The second clade is composed of the hermaphroditic, polygamous, and gynodioecious species of the subgenus *Acetosa* as well as the dioecious African species of *Acetosa, R. sagittatus*. The only species of the subgenus *Platypodium* (*R. bucephalophorus*) appears here as a basal species to these two clades. Finally, the species of the subgenus *Rumex* belong to the third clade. A similar topology reflected a MP strict consensus tree (consistency index [CI] = 0.65; retention index [RI] = 0.68; 805 steps; four equally parsimonious trees), although the tree was not completely resolved in this case, given that the dioecious, the polygamous-gynodioecious clade and *R. bucephalophorus* formed a polytomy. To minimize the effects of homoplasy, we reweighted the characters by the maximum value of rescaled consistency indices, which resulted in a single MP tree (CI = 0.87; RI = 0.88; 365 steps), as in figure 2, with *R. bucephalophorus* being a basal species to the two clades.

The length of the trnL intron varied between 413 and 640 bp due primarily to a highly variable internal region composed of microsatellite-like sequences that were omitted because they could not be reliably aligned. The length was about 370 bp for the trnL-trnF intergenic spacer. We combined these sequences into a single data set. Therefore, the alignment data set contained 738 characters, 51 of which were informative for parsimony analysis. NJ (Tamura distance), MP strict consensus (CI = 0.94; RI = 0.96; 176 steps; 468 equally parsimonious trees), and ML (K81uf model of DNA substitution) trees reflected the same topology, which was similar to that found for the ITS sequences (fig. 3). A major difference between the two trees of figures 2 and 3 was the position of *R. bucephalophorus*, which in the chloroplastidial tree appears as a basal species to the dioecious clade but which appeared as a basal species in the ITS tree for both the dioecious and the polygamous-gynodioecious clades. Despite the discrepancy between the phylogenetic position of *R. bucephalophorus* in the ITS and chloroplast trees, the bootstrap values for the chloroplast tree and the data on the chromosome numbers both suggest that *R. bucephalophorus* is basal to the dioecious clade. The internal bootstrap values separating the three clades are

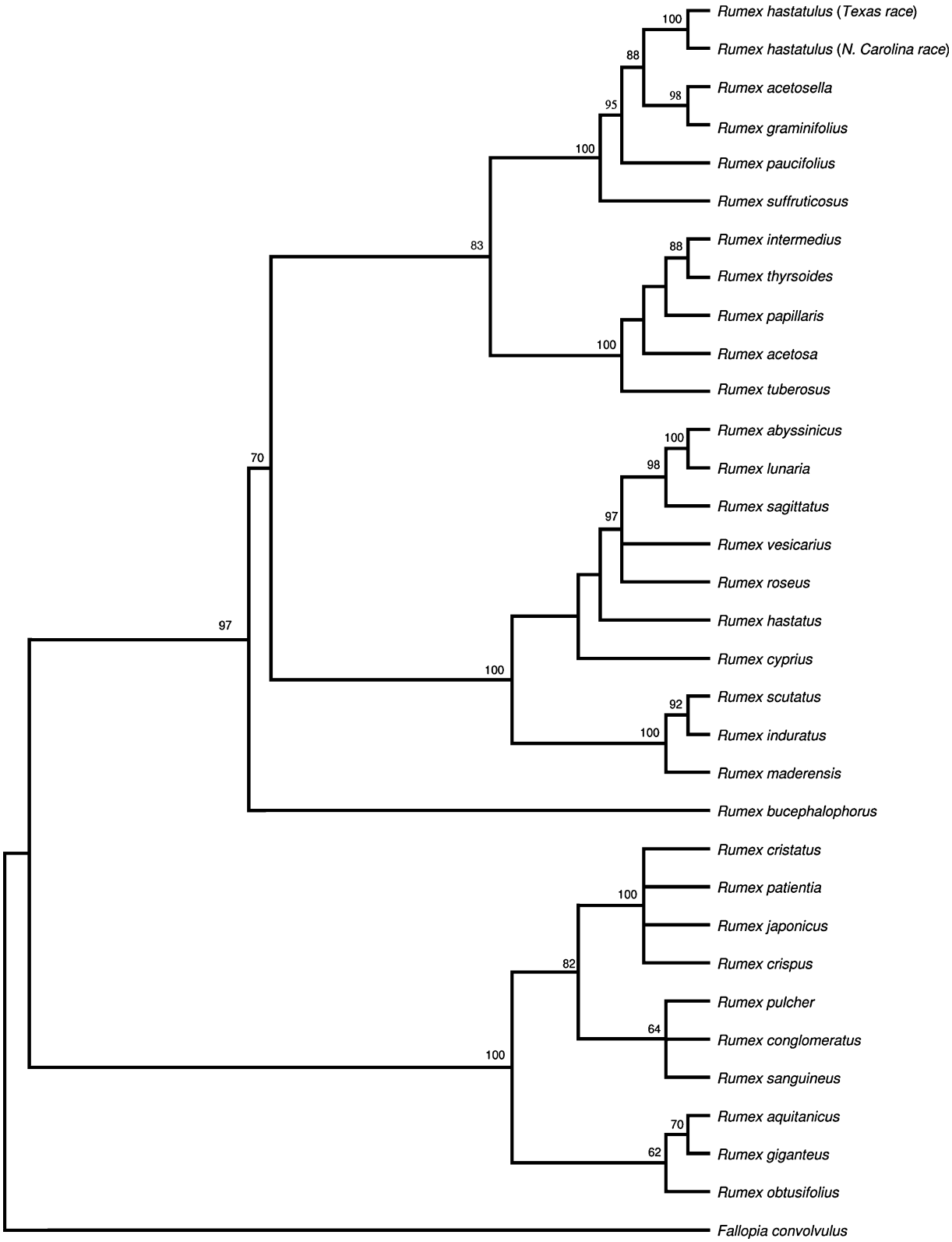


FIG. 2.—Maximum likelihood tree constructed for the *Rumex* species using the ITS sequences. Numbers at each node indicate bootstrap support values. *Fallopia convolvulus* (Polygonaceae) was used as an out-group species.

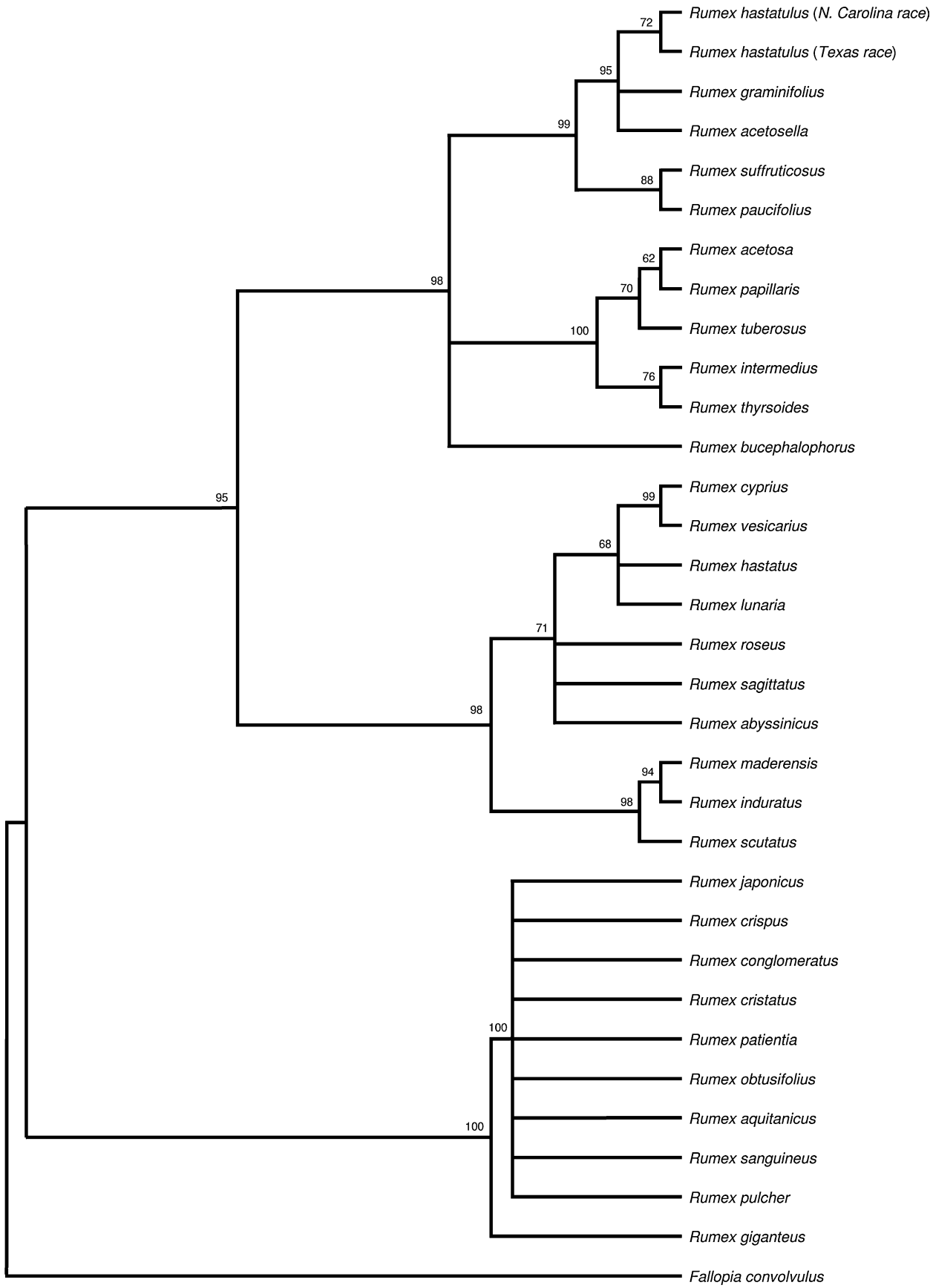


FIG. 3.—Maximum likelihood tree constructed for *Rumex* species using the chloroplastidial sequences.

overall higher on the chloroplast tree (compare figs. 2 and 3), and *R. bucephalophorus* shares the ancestral chromosome number of the dioecious clade (table 1 and fig. 4).

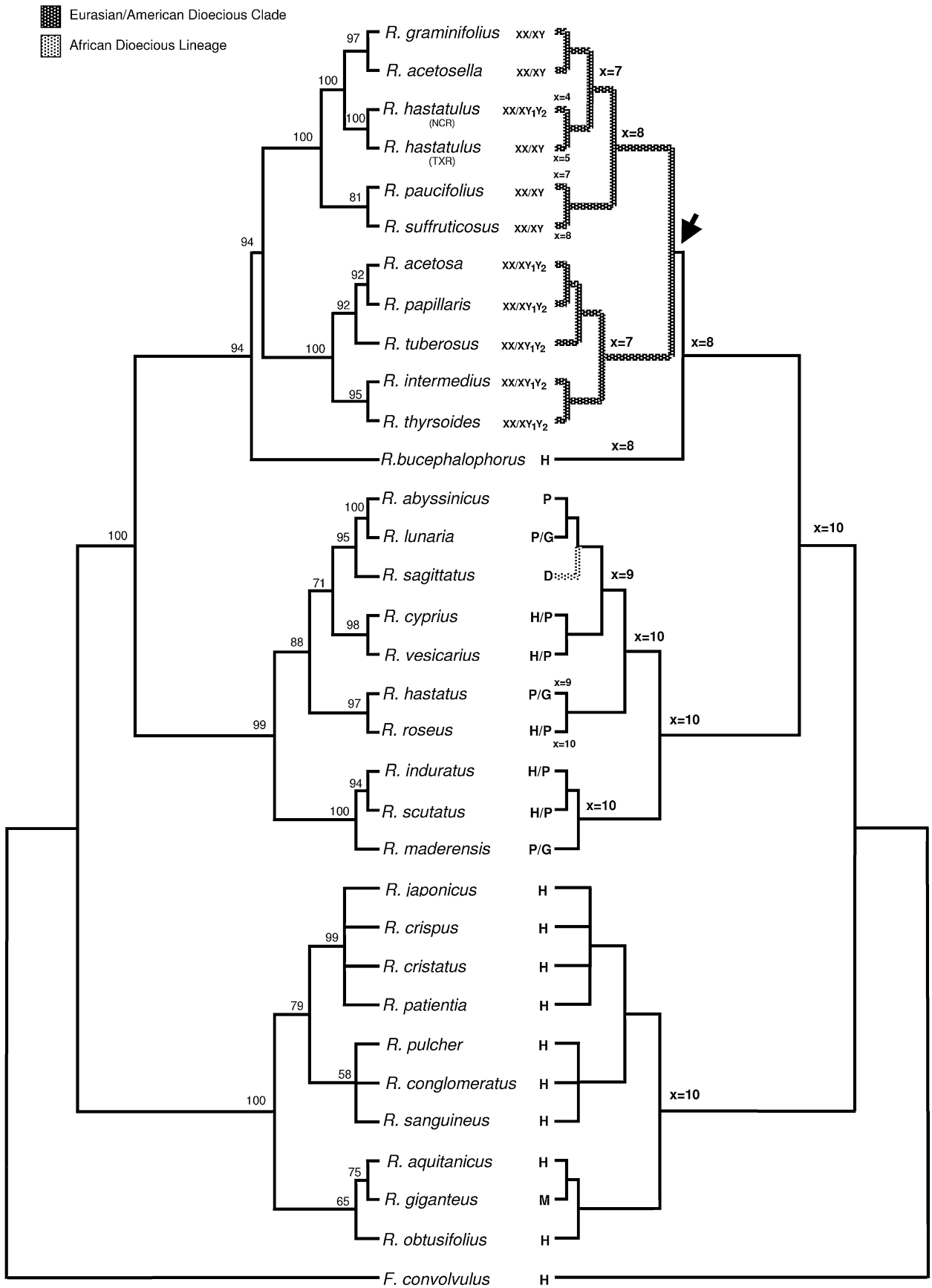
Next, we combined the chloroplastidial and the nuclear sequences into a single data set. Consistency between the nuclear and chloroplast data sets was found after conducting an ILD partition-homogeneity test ($P = 0.06$; $P = 0.08$ when excluding invariant characters according to Lee [2001]). As shown in figure 4, we found better-resolved trees with the combined data using NJ (Tamura-Nei gamma distance), ML (HKY + G model of DNA substitution), and MP approaches (CI = 0.69 [0.89]; RI = 0.74 [0.91]; 995 [516] steps; a single most parsimonious tree; in brackets, values when characters were reweighted by maximum value of rescaled consistency indices). The tree of figure 4 reveals three clades, which are supported by high bootstrap values. Figure 4 also shows the correlation between the resulting molecular phylogeny and the evolution of the mating systems in the genus *Rumex*. Thus, one clade grouped all Eurasian and American dioecious species with *R. bucephalophorus*—the hermaphroditic species of the subgenus *Platypodium*. Dioecious species were divided into two subclades: one subclade composed of the species having an XX/XY sex-chromosome system such as *R. acetosella* and *R. graminifolius* from the subgenus *Acetosella* and *R. suffruticosus, R. paucifolius*, and *R. hastatulus* (which includes two allopatric chromosomal races, XX/XY and XX/XY_1Y_2) from the subgenus *Acetosa*; and the second subclade composed of the rest of the dioecious species of the section *Acetosa* of subgenus *Acetosa* with an XX/XY_1Y_2 chromosome system. The second clade was composed of the hermaphroditic, polygamous, and gynodioecious species of the subgenus *Acetosa*, as well as the African dioecious species, *R. sagittatus,* of the subgenus *Acetosa*. The third clade includes strictly hermaphroditic species of subgenus *Rumex*. Within *Rumex*, however, the endemic Hawaiian species *R. giganteus* is monoecious.

The new phylogeny also reflects the evolution of the chromosome number in the genus (fig. 4). The clade composed of hermaphroditic species of the subgenus *Rumex* is made up of species with a basic number of x = 10. The clade composed of polygamous and gynodioecious species of the subgenus *Acetosa* is composed of species with a basic chromosomal number of x = 10 or x = 9. Finally, the clade leading to *R. bucephalophorus* and to the dioecious species is composed of species with x = 8 (*R. bucephalophorus* and *R. suffruticosus*) and with x = 7 (the rest of dioecious species). An additional reduction of the basic chromosomal number was found in *R. hastatulus*, with two races, the Texas race with x = 5 and the North Carolina race with x = 4. All the chromosome data were gathered from the literature except for *R. suffruticosus*, a dioecious species for which no cytogenetic analyses are available. Our analysis of meiotic cells showed that this species has a basic chromosome number of x = 8 with a pair of heteromorphic sex chromosomes in the males resembling those found in *Silene latifolia* and in *R. hastatulus,* the Texas race (data not shown). We have thus assumed that *R. suffruticosus* has an XX/XY chromosome system, and that, as in *R. acetosella* and in the Texas race of *R. hastatulus,* an active male-determining Y regulates sex.

Discussion

The genus *Rumex* is currently classified into four subgenera, *Acetosella, Acetosa, Rumex*, and *Platypodium* (Rechinger 1937, 1964; Löve 1944; Löve and Kapoor 1967; Degraeve 1976). Some authors (Löve and Kapoor 1967) have even proposed dividing it into four genera: *Rumex* L., *Acetosa* Mill., *Acetosella* (Meissn.) Fourr., and *Bucephalophora* Pau (subgenus *Platypodium*). This classification into four groups of species is based mainly on a few imprecise morphological characters, such as leaf and valve morphology, which do not provide a set of differential characters for consistently separating *Rumex* species into four groups. In fact, our molecular phylogenetic analysis supports a different classification of the genus *Rumex*. We found three phylogenetic clades within *Rumex*, implying a revision of the systematics for this genus. Thus, the subgenus *Rumex* appears coherent since all their species appear to form a well-supported (100% bootstrap) clade of closely related species. However, we find no evidence to maintain the subgenera *Platypodium* and *Acetosella*. Thus, *R. bucephalophorus*, as the only representative species of the subgenus *Platypodium*, is closely related to all the Eurasian and American dioecious species of subgenera *Acetosa* and *Acetosella*. On the contrary, our phylogeny finds support to include the species of these two subgenera within *Acetosa* and to separate this subgenus into two groups: one including *R. bucephalophorus* together with all the Eurasian and American dioecious species of the subgenera *Acetosa* and *Acetosella*; and a second group which would include the hermaphroditic-polygamous and the polygamous-gynodioecious species of the subgenus *Acetosa* together with the African dioecious species *R. sagittatus* (also, subgenus *Acetosa*). Notably, the cladistic features found within the ITS sequences are also in agreement with this last conclusion (fig. 1).

The new phylogeny is consistent with a classification of *Rumex* species based on their basic chromosome number (fig. 4). Similarly, Smith (1969), following a karyoevolutionary approach, proposed a single origin for all dioecious species for which x = 7 might be the basic chromosome number, this number being derived in the distant evolutionary past by a mechanism of chromosome number reduction from a higher basic chromosome number. Implicitly, both our system and that of Smith imply that the phylogeny of the genus *Rumex* might be inferred from the cytogenetic data, which indicate that the evolution of *Rumex* species followed a process of chromosomal reduction from x = 10 as a base chromosome number toward x = 7 through intermediate stages (x = 9 and x = 8). Our phylogeny supports the contention that the basic ancestral chromosome number might be x = 10 and that this number persisted in all hermaphroditic species of the subgenus *Rumex* as well as in the polygamous species of the section *Scutati* of the subgenus *Acetosa* (*Rumex scutatus, Rumex induratus*, and *Rumex roseus*) and *Rumex maderensis* (section *Hastati*). These latter species form a clade with the polygamous species with x = 9 (*Rumex abyssinicus, Rumex vesicarius*, and


Eurasian/American Dioecious Clade
African Dioecious Lineage
R. graminifolius XX/XY
R. acetosella XX/XY
R. hastatulus (NCR) XX/XY1Y2
R. hastatulus (TXR) XX/XY
R. paucifolius XX/XY
R. suffruticosus XX/XY
R. acetosa XX/XY1Y2
R. papillaris XX/XY1Y2
R. tuberosus XX/XY1Y2
R. intermedius XX/XY1Y2
R. thyrsoides XX/XY1Y2
R.bucephalophorus H
R. abyssinicus P
R. lunaria P/G
R. sagittatus D
R. cyprius H/P
R. vesicarius H/P
R. hastatus P/G
R. roseus H/P
R. induratus H/P
R. scutatus H/P
R. maderensis P/G
R. japonicus H
R. crispus H
R. cristatus H
R. patientia H
R. pulcher H
R. conglomeratus H
R. sanguineus H
R. aquitanicus H
R. giganteus M
R. obtusifolius H
F. convolvulus H
x=4
x=5
x=7
x=8
x=9
x=10

Rumex cyprius) and with the polygamous-gynodioecious species *R. hastatus* and *Rumex lunaria* also with x = 9. *Rumex sagittatus*, a dioecious species, has a chromosomal number of x = 9, lacks differentiated sex chromosomes, and is included within the section *Afroacetosa* of the subgenus *Acetosa* (Degraeve 1976). Our data support the contention that *R. sagittatus* is phylogenetically related to other x = 9 polygamous and gynodioecious species and that it might be considered an advanced state in the evolutionary pathway from polygamy to gynodioecy and then to dioecy. The third clade appears to be composed of two species, one hermaphroditic and one dioecious, with x = 8, and of the Eurasian and American dioecious species, with x = 7. Therefore, it appears that a process of chromosome reduction accompanies the evolution toward the gynodioecy and dioecy in *Rumex*. A further process of chromosome number reduction has occurred in *R. hastatulus*, a dioecious species with x = 5 (Texas race) or x = 4 (North Carolina race). In the case of the North Carolina race, the reductional process involved an autosome-X translocation, which led to the XX/XY_1Y_2 sex-chromosome system in this species (Smith 1964, 1969).

In this sense, our data support the idea that a common ancestor led to all the Eurasian and American dioecious species of *Rumex*. This view contradicts that offered by the current morphological classification of the genus, which assumes that dioecy has appeared independently several times in *Rumex*. Also, we find that the common ancestor leading to all *Rumex* species must be hermaphroditic. However, our data also suggest that gynodioecy might have played a major role in the evolutionary pathways between the ancestral hermaphroditic species and the current dioecious species because a second different lineage could be evolving from hermaphroditism towards dioecy via gynodioecy in Africa. In fact, we have demonstrated that in a clade of related Eurasian and African species, some have the ancestral basic chromosomal number (x = 10) while others have intermediate chromosomal numbers (x = 9). This group is formed by polygamous, gynodioecious, and incipient dioecious species. Dioecy has evolved at least twice in the genus *Silene* as a state derived from gynodioecy (Desfeux et al. 1996), although hermaphroditism might be ancestral to gynodioecy. It is likely that dioecy has several independent origins in *Ribes* (Senters and Soltis 2003). In the Siparunaceae family, dioecy has also evolved repeatedly, but in this case from monoecy (Renner and Won 2001). However, it appears that monoecy is not likely to be a transitory condition toward dioecy, at least in monocotyledons(Weiblen, Oyama, and Donoghue 2000). In our analysis, we have included one exceptional species of the subgenus *Rumex*, the monoecious *R. giganteus*. It appears that breeding systems in this and other endemic Hawaiian species (such as *Rumex albescens* or *Rumex skottbergii*) have evolved towards monoecy. In fact, other species such as *Rumex obtusifolius*, a strictly hermaphroditic species of this subgenus, have evolved towards monoecy also in Hawaii populations (Wagner, Herbst, and Sohmer 1999). It appears then that in these insular populations the breeding systems follow different evolutionary paths with respect to the rest of the *Rumex* species.

Within the main clade of dioecious species, there are two subclades. One of the subclades is composed of the XX/XY species, which includes the Eurasian species *R. suffruticosus*, an endemic species of the Iberian Peninsula, *R. acetosella* and its relative *R. graminifolius*, and the American dioecious species *R. paucifolius* and *R. hastatulus*. The other subclade is composed of the species *R. acetosa* and its relatives, which form a homogeneous group of species characterized by similar morphological and karyological characteristics, including an XX/XY_1Y_2 sex-chromosome system (Degraeve 1976; Wilby and Parker 1988). It is worth mentioning here that within the American species of the subgenus *Acetosa*, *R. paucifolius* has been considered a close relative of *R. acetosella*, while *R. hastatulus* has been described as a close relative of the *R. acetosa* complex group (Smith 1968, 1969). However, according to our phylogenetic scheme, both *R. hastatulus* and *R. paucifolius* share a common ancestor with *R. acetosella* and *R. graminifolius*. In fact, *R. acetosella–R. graminifolius* and *R. hastatulus* appear to be the closest relatives in this group.

In other plant taxa, it has been found that dioecy is a relatively recent event (Desfeux and Lejeune 1996; Desfeux et al. 1996; Guttman and Charlesworth 1998). Filatov et al. (2000), taking a mean rate of change in plant nuclear DNA of 0.6% per site per million years (Gaut 1998), roughly estimated that the origin of dioecy in *Silene* might be between 10–20 MYA. We are prompted here to estimate, at least roughly, whether dioecy appeared also as an only relatively recent event. Because distance methods suggested rate variation across some lineages rather than constancy, we reconstructed divergence times in the absence of a molecular clock. For this purpose, we selectively pruned away lineages that deviated significantly from rate constancy in a series of relative-rate tests using the program LINTRE (Takezaki, Rzhetsky, and Nei 1995). Once heterogeneous sequences were eliminated, we constructed a linearized tree under the assumption of rate constancy and reestimated the branch lengths (Takezaki, Rzhetsky, and Nei 1995). Using the estimated mean rate of change in plant nuclear DNA of 0.6% per site per million years, the ITS mean distance–corrected estimates between clades suggest that dioecy appeared in *Rumex* between 15–16 MYA, while the divergence time between the *R. acetosella–R. suffruticosus* clade and the *Acetosa* clade should be 12–13 MYA.

←

FIG. 4.—Left side: maximum likelihood tree constructed for *Rumex* species using chloroplastidial and nuclear sequences combined into a single data set. Numbers at each node indicate bootstrap support values. Right side: correlation between the molecular phylogeny and the evolution of both the basic chromosome numbers (x) and the mating systems in the genus *Rumex*. Both the Eurasian/American dioecious clade and the African dioecious lineage are marked by thicker branch. The arrow labels the dioecious node. H, hermaphrodite; P, polygamous; G, gynodioecious; M, monoecious; D, dioecious; NCR, North Carolina race of *R. hastatulus*; TXR, Texas race of *R. hastatulus*.

The establishment of a phylogeny in *Rumex* has proved to be informative for the analysis of the evolution of sex-chromosome and sex-determination mechanisms in the genus. It has been commonly accepted that the multiple Y-chromosome system of the *Rumex* species of the Acetosa group might represent a derived state from an XX/XY ancestor in the group, and our data support this view. On these grounds, two hypotheses have been proposed to explain the origin of the XX/XY_1Y_2 system. One hypothesis suggests that the multiple sex-chromosome system might have been originated through a chromosomal translocation between one member of a pair of autosomes and the ancestral X chromosome in an XX/XY species. This case has been proposed to occur in *R. hastatulus* for which the chromosome number reduction that occurred between the Texas race and the North Carolina race involved an autosome-X translocation, which led to the apparition of the XX/XY_1Y_2 sex-chromosome system in this species (Smith 1969). The alternative hypothesis suggests that the appearance of two Y chromosomes might be due to a process of misdivision of an ancestral Y chromosome as has been proposed for *R. acetosa* and related species (Ruiz Rejón et al. 1994). The first hypothesis is obviously acceptable for explaining the appearance of the multiple sex-chromosome system in the North Carolina race of *R. hastatulus*, and, consequently, it appears most plausible to explain the emergence of the XX/XY_1Y_2 system in *R. acetosa* and relatives. In this latter case, the translocation hypothesis involves the chromosome number reduction from $x = 8$ to $x = 7$ for the appearance of the two Ys, while the hypothesis of the misdivision points to a dioecious ancestor with $x = 7$ chromosomes. Unfortunately, the phylogenetic data gathered here do not provide strong support to discriminate between these two hypotheses in the section *Acetosa*, given that the hypothetical XX/XY dioecious ancestor of the *Acetosa* group of species with XX/XY_1Y_2 could have had either $x = 7$ or $x = 8$ chromosomes, two chromosome numbers found in the sister group of dioecious species with an XX/XY system (fig. 4).

In relation to sex-determination mechanisms, Westergaard (1958) proposed that the X/A balance mechanisms have evolved secondarily from male-determining Y-chromosome mechanisms. The X/A balance in sex determination precludes the occurrence of polyploidy (Muller 1925), and, in cases of demonstrated polyploidy, sex appears to be controlled by a Y-based determination mechanism (Smith 1969). The genus *Rumex,* thus, is of particular interest in this connection because among its dioecious representatives both modes of sex determination occur in a variety of different species. We have analyzed five species with an X/A sex-determination mechanism (the *Acetosa* group of diploid species: *R. acetosa, Rumex papillaris, Rumex tuberosus*, *Rumex thyrsoides*, and *Rumex intermedius* and four species with a Y-based sex-determination mechanism: *R. acetosella* and *R. paucifolius* for which polyploid populations have been described; *R. graminifolius*, which is hexaploid; and the Iberian endemism *R. suffruticosus*, for which the only known cytogenetic data are those found by us in this study ($x = 8$; XX/XY) and for which we could assume an Y-based sex-determination mechanism. The tree topology determined in this paper shows the X/Y species group as a sister group to the X/A species group. Within the X/Y group of species, the North Carolina race of *R. hastatulus* has evolved secondarily from the X/Y mechanism to the X/A mechanism. Curiously, the same evolutionary change from the XX/XY (Y-based sex-determination mechanism) to the XX/XY_1Y_2 (X/A sex-determination mechanism) sex-chromosome system appears to have occurred independently in the two lineages, one in the ancestor of the Eurasian *R. acetosa* and its relatives and the other in the American species related to *R. acetosella* (*R. hastatulus*), as proposed earlier by Smith (1969) and Degraeve (1976). Our data support the idea that the X/A mechanism is a derived situation of the Y-based mechanism. Circumstantial evidence supporting this assumption was provided by the fact that the X/Y sex-determination mechanism is taxonomically far more widely distributed than X/A (Charlesworth 1996). However, our results now give direct evidence supporting the contention that the X/Y mechanism is indeed primitive in the genus *Rumex*.

In conclusion, as a response to the questions considered above, our study demonstrates (1) that all Eurasian and American dioecious species of *Rumex* have a single origin and that gynodioecy may have been an intermediate state on the way to dioecy, as appears to be occurring in African species; (2) that the Y-based sex-determination mechanism is older than the X/A mechanism and also that the multiple sex-chromosome system derived from an XX/XY system; (3) and that a new infrageneric classification can be proposed for *Rumex* species consistent with their mating system and with their karyotype evolution.

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