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

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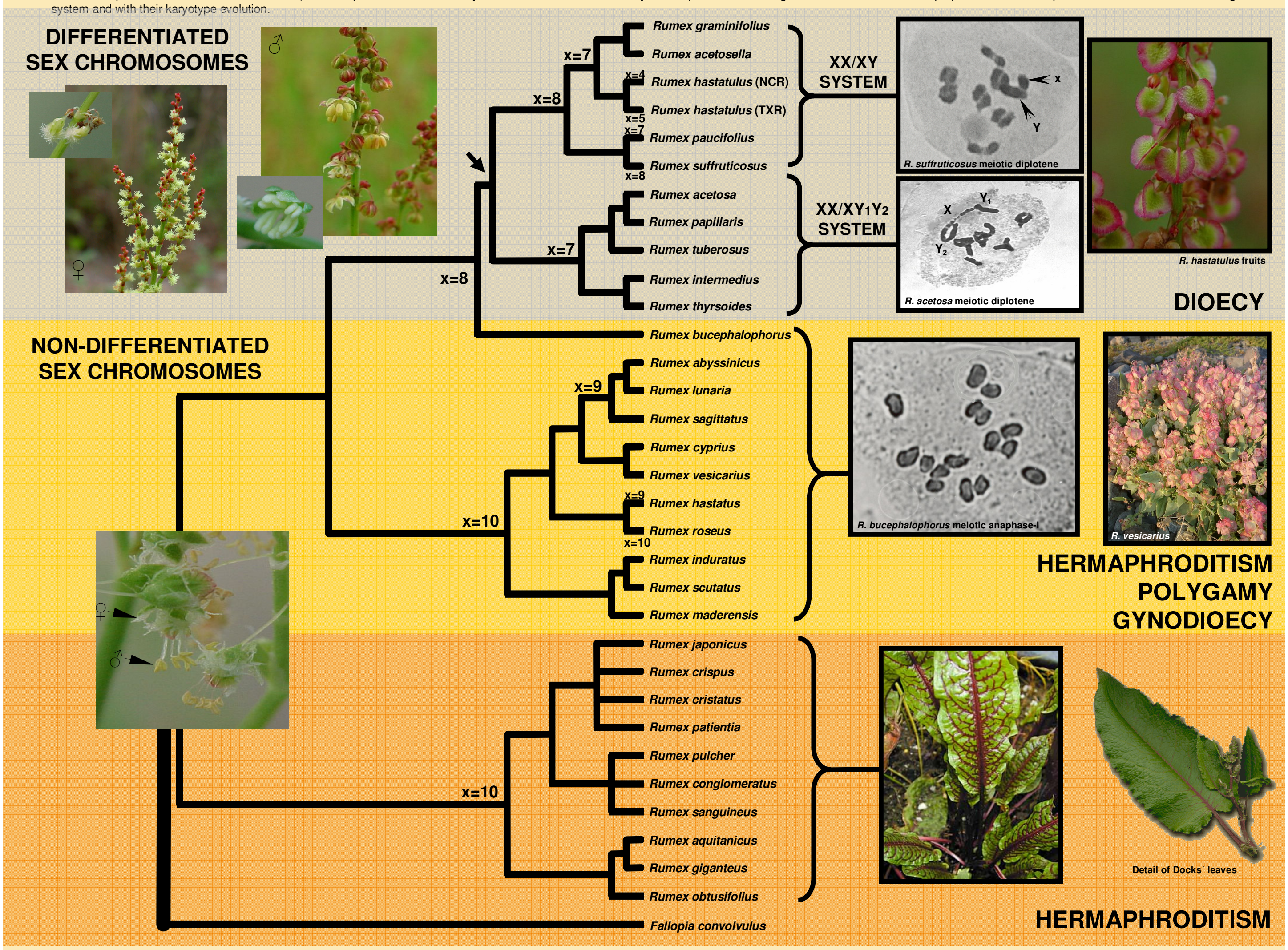
INTRODUCTION

The genus *Rumex* represents an exceptional case to test hypothesis regarding sex-chromosomes origin and evolution that still remain puzzling. In fact, *Rumex* constitutes a big group of species in which almost every mating system is present, comprising of hermaphrodite, polygamous, gynodioecious, monoecious and dioecious representatives. Here we present an evolutionary picture of the genus concerning basic chromosome number, sex-chromosomes and mating-systems evolution.

The genus *Rumex* is currently divided into four subgenera (Table 1): *Acetosella*, *Acetosa*, *Platypodium*, and *Rumex*. *Acetosella* contains two species, *R. acetosella* (which has several subspecies) and *R. 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. Within the subgenus *Acetosa*, the section *Acetosa* is composed of *R. acetosa* and its relatives, which form an homogeneous group of species characterized by similar morphological and karyological characteristics, including a XX/XY_1Y_2 sex-chromosome system plus a sex-determination mechanism based on the X/A balance. However, within the section *Americanae* of the subgenus *Acetosa*, there are two species: *R. paucifolius*, which has the XX/XY system; and *R. hastatulus*, which has two chromosomal races, one with the XX/XY (called the "Texas race") and the other with the XX/XY_1Y_2 (called the "North Carolina" race). 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 comprised of hermaphroditic and polygamous species. Strikingly, *Scutati* has a dioecious species, *R. suffruticosus*, for which no chromosomal data were available until now. The sections *Hastati* and *Afroacetosa* are comprised of polygamous and gynodioecious species as well as a dioecious one, *R. sagittatus,* which lacks differentiated sex chromosomes. Meanwhile, the third subgenus, *Platypodium*, has one species (and several subspecies), *R. bucephalophorus,* which is hermaphroditic. Finally, the subgenus *Rumex* is composed of hermaphroditic species, although endemic Hawaiian species such as *R. giganteus* have evolved towards monoecy.

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; also this classification involves that there has been no evolutionary constraint on the evolution of sexual systems and that forward and reverse evolution occur with equal probability. We test here whether these assumptions are correct or not by means of a phylogenetic analysis of the genus *Rumex* based on one nuclear and one chloroplastidial marker (Table 1). Specifically, we want to address whether: i) dioecy has appeared once or several times in *Rumex*; ii) the Y-based sex-determination mechanism precedes to the X/A mechanism; iii) the multiple sex-chromosome system derived from an XX/XY system; iv) a different infra-generic classification can be proposed for *Rumex* species consistent with their mating system and with their karyotype evolution.


DIFFERENTIATED SEX CHROMOSOMES
♂
♀
x=7
x=8
x=4
x=5
x=7
x=8
x=7
x=8
Rumex graminifolius
Rumex acetosella
Rumex hastatulus (NCR)
Rumex hastatulus (TXR)
Rumex paucifolius
Rumex suffruticosus
Rumex acetosa
Rumex papillaris
Rumex tuberosus
Rumex intermedius
Rumex thyrsoides
XX/XY SYSTEM
XX/XY1Y2 SYSTEM
x
Y
X
Y1
Y2
R. suffruticosus meiotic diplotene
R. acetosa meiotic diplotene
R. hastatulus fruits
DIOECY
NON-DIFFERENTIATED SEX CHROMOSOMES
Rumex bucephalophorus
x=9
Rumex abyssinicus
Rumex lunaria
Rumex sagittatus
Rumex cyprius
Rumex vesicarius
x=9
Rumex hastatus
Rumex roseus
x=10
x=10
Rumex induratus
Rumex scutatus
Rumex maderensis
R. bucephalophorus meiotic anaphase-I
R. vesicarius
HERMAPHRODITISM
POLYGAMY
GYNODIOECY
♀
♂
Rumex japonicus
Rumex crispus
Rumex cristatus
Rumex patientia
Rumex pulcher
Rumex conglomeratus
Rumex sanguineus
Rumex aquitanicus
Rumex giganteus
Rumex obtusifolius
x=10
Fallopia convolvulus
Detail of Docks´ leaves
HERMAPHRODITISM

Species	S.D.	X	Procedence	Accession Numbers
<u>Subgenus Acetosa</u>				
Sec. 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, Sª Baza, Granada (Spain) WM	AJ583841, AJ583854, AJ580775, AJ580791
Rumex tuberosus	D (XX/XY_1Y_2)	7	Jabal, Sinjar (Iraq) VSC-, Omar, S., Kaisi, A.L. & Khayat, A.L. 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
Sec. Americanae				
Rumex hastatulus (NCR)	D (XX/XY_1Y_2)	4	Cumberland County, North Carolina (USA) WM	AJ698484, AJ699268, AJ704987, AJ704988
Rumex hastatulus (TXR)	D (XX/XY)	5	Masan County, Texas (USA) WM	AJ698485, AJ699269, AJ810979, AJ810989
Rumex paucifolius	D (XX/XY)	7	Challis National Forest, Custer County, Idaho (USA) VSC.- Steele, R. 702 BOISE.	AJ889018, AJ889019, AJ890454, AJ890455
Sec. Scutati				
Rumex scutatus	H/P	10	Sª 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
Sec. 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
Sec. 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-, Dellon, A. ARH3094 K	AJ810935, AJ810945, AJ810981, AJ810991
Sec. Afroacetosa				
Rumex abyssinicus (SsA)	P	9	Gilo, Imatong Mts. (Sudan) VSC.- Friis, I. & Vollesen, K. 87 K	AJ698487, AJ699271, AJ844278, AJ844279
Rumex sagittatus (SsS)	D	9	Montagu Island, New South Wales (Australia) VSC-, Heyligers, P.C. 89028 K	AJ698486, AJ699270, AJ889014, AJ889015
<u>Subgenus Acetosella</u>				
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-, Rebristaya, O. 6315 K	AJ810934, AJ810944, AJ831539, AJ844277
<u>Subgenus Rumex</u>				
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	Mr. Nigel Huneyman personal collection. C	AJ810936, AJ810946, AF338220*
Rumex giganteus	M	10	Pohakoloa, Hawaiian Islands (USA). Hawaiian Plant DNA Library (HPDL). University of Hawaii at Manoa (USA).	AJ698482, AJ699266, AJ810982, AJ810992
<u>Subgenus Platypodium</u>				
Rumex bucephalophorus	H	8	Padul, Granada (Spain) WM	AJ583846, AJ583859, AJ580783, AJ580787
Fallopia convolvulus	H	10	Capileira, Granada (Spain) VSC.-Molero, J. GDA. 10719.	AJ583845, AJ583858, AF040064*

Table 1. List of analyzed *Rumex* species.

• **Notes to:**
- **SPECIES:** *(NCR).*- North Carolina Race; *(TXR).*- Texas Race; *(SsA).*- Subsection Abyssinici; *(SsS).*- Subsection Sagittati.
- **SEX-DETERMINATION SYSTEMS** *(S.D.)*: *(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, USA; *(DUKE).*- Duke University Herbarium, USA; *(GDA).*- University of Granada Herbarium, Spain; *(K).*- Royal Botanic Gardens, Kew, UK;
- **ACCESSION NUMBERS:** Intron, spacer, ITS1 and ITS2 except for * (Intron, spacer, ITS1-2).

RESULTS AND CONCLUSIONS

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*). Within the main clade of dioecious species, there are two subclades. One of the subclades is composed by the XX/XY species, which includes the Eurasian species *R. suffruticosus*, an endemic species of the Iberian Peninsula (for which we have described a XX/XY system), *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.

This new classification is consistent with the evolution of mating system and sex determination of *Rumex* species and of the karyotype. In contrast to the current view, this new phylogeny suggest a common origin for all Eurasian and American dioecious species of *Rumex* with gynodioecy as an intermediate state on the way to dioecy, since a second different lineage could be evolving from hermaphroditism towards dioecy via gynodioecy in Africa. 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. Also, 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 (hermaphroditic species) toward x=7 (dioecious species) through intermediate stages with x=9 and x=8 chromosomes (polygamous and gynodioecious species).

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