

# Karyotype evolution supports the molecular phylogeny in the genus *Ranunculus* (Ranunculaceae)



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## ABSTRACT

Karyotype evolution can be reconstructed by the characterization of chromosome morphology, based on the position of centromeres. Different karyotypes often reflect speciation events within phylogenies as they can establish crossing barriers between species. Hence, evolution of karyotypes often is congruent with splits and differentiation of clades within phylogenies of angiosperm genera. Here we study karyotype evolution in the big cosmopolitan genus *Ranunculus* and in related genera to test the hypothesis that karyotypes are congruent with major clades. We investigated karyotypes on mitoses of 36 species, evaluated literature records for additional 87 species, and reconstructed ancestral states by mapping karyotypes onto a published molecular phylogenetic tree. Altogether ten karyotypes can be discriminated as character states, eight based on the base number  $x=8$ , and two on the base number  $x=7$ . The ancestral type within *Ranunculus* is characterized by four metacentric and four submetacentric/subtelocentric chromosomes, and dominates in five major basal clades of *Ranunculus* (*R.* subg. *Auricomus*) and in four of the related genera (*Coptidium*, *Halerpestes*, *Kumlienia*, and *Trautvetteria*). Another four karyotypes are ancestral and predominant in two major clades within *Ranunculus* (*R.* subg. *Ranunculus*), while three further ones occur only on terminal branches of the buttercup phylogeny. Among related genera, *Ficaria* and *Ceratocephala* also show derived karyotypes. Karyotype morphology thus supports generic and infra-generic classifications based on molecular and morphological data. Karyotype evolution drives in general to increasing number of chromosomes with asymmetric arms, and to a reduction from  $x=8$  to  $x=7$  chromosomes. A review of interspecific homoploid hybridization in sympatric species, and of crossing experiments suggests enhanced crossability of species with the same karyotype and strong crossing barriers between those with different karyotypes. We conclude that karyotype evolution is a major driver of speciation and differentiation of clades within *Ranunculus*.

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## 1. Introduction

Chromosomes within a taxon (e.g. family, genus or even species) may vary concerning their number (including ploidy level and aneuploidy) but may also show variation in absolute (in  $\mu\text{m}$ ) and relative chromosome size (percentage of the total length of the karyotype), in chromosome morphology, and in staining properties (Sharma and Sen, 2002). Morphological characters of chromosomes are mostly due to the position of the centromere (Levan et al., 1964). The description of chromosome morphology is a powerful method to characterize genomes in plants and in animals as well (including

humans). Karyological data are essential information and provide important characters for plant systematics and evolutionary analysis (Stace, 2000; Crawford et al., 2005). In combination with molecular phylogenies, karyotype evolution gives insight into evolution of species in radiations (e.g. Weiss-Schneeweiss et al., 2008). Karyotype features represent an important aspect for plant speciation as chromosomal differences establish immediate postzygotic crossing barriers (e.g. Rieseberg, 1997). Under this aspect, evolution of karyotypes is expected to be congruent with splits and differentiation of clades within phylogenies of angiosperm genera (e.g. Blöchl et al., 2009). Extant species with divergent karyotypes would not be able to cross, while species with the same karyotype should be able to produce hybrids.

The genus *Ranunculus* comprises about 600 species with a cosmopolitan distribution (Tamura, 1995). It is the largest genus of the family Ranunculaceae and varies broadly in morphology, ecology,

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and life cycle. It is distributed mainly in submeridional to temperate zones of both hemispheres as well as in high mountain systems. The genus has been and is still subject of investigations of evolutionary history (Emadzade et al., 2010, 2011, 2015; Hoffmann et al., 2010; Hörandl et al., 2005, 2009; Johansson, 1998; Paun et al., 2005, 2008), and well-supported phylogenetic trees are available (Emadzade et al., 2011; Hörandl and Emadzade, 2012). As the genus *Ranunculus* shows pronounced karyological variability we check the homogeneity of karyotypes within the clades in the phylogenetic tree using our own data as well as data from the literature. Hybridization is frequent within the genus, but not evenly distributed across the phylogeny (Hörandl et al., 2005; Hörandl, 2014). Floral features of buttercups are quite uniform (Hörandl and Emadzade, 2012) and the species are lacking pollinator specificity (Steinbach and Gottsberger, 1995); hence, we assume that karyotype evolution, beside other isolation mechanisms, played an important role in the establishment of crossing barriers among ancestors and extant members of the major clades.

Previous hypotheses about ancestral vs. derived karyotypes within the genus, as e.g. proposed by Goepfert (1974), have remained speculative without a solid phylogenetic reconstruction for the whole genus. By reconstructing ancestral states of karyotypes on a recently published molecular phylogeny based on plastid and nuclear markers, we want to get insights into the karyotype evolution of the genus and test the hypothesis of a congruence of clades with karyotypes. Furthermore, we discuss the possible role of karyotype differentiation for establishment of crossing barriers by comparing patterns of hybridization of extant species within and between clades of *Ranunculus*.

## 2. Material and methods

### 2.1. Plant material and names

We present here data for altogether 123 species of Ranunculaceae. The nomenclature for plant names follows Hörandl and Emadzade (2012); in a few cases the names from “Flora Europaea” (Tutin and Akeroyd, 1993), “Flora Hellenica” (Strid, 2002), “Flora Palaestina” (Zohary, 1966) or “Flora of Turkey” (Davis, 1965) are added. The identification numbers refer to the herbarium specimens deposited in Z + ZT. The origin of the karyologically investigated plants is given in the electronic Appendix 1.

### 2.2. Chromosome number

Karyological investigations on 36 *Ranunculus* species have been carried out on root tips of cultivated plants (for methods see Baltisberger and Widmer, 2009a). Five to ten metaphases were counted for each individual to determine chromosome numbers, mostly several (at least two) individuals were investigated per site. The numbers of investigated individuals per site are indicated in Table 1. Chromosome numbers in the literature were checked with Goldblatt and Johnson (1979+). To allow the inclusion of the huge amount of literature data for this big genus, we focused here on karyomorphological data only. We did not include data from molecular cytogenetic analyses because this may have strongly limited species sampling.

### 2.3. Chromosome size

The chromosome size depends on the method of preparation (e.g. chemicals, duration of pretreatment), the taxon investigated, and the person performing the investigation (experience, technique of squashing the root tips, etc.). Therefore, absolute chromosome size is of reduced value but can be used as character of given chromosomes in special cases (e.g. bimodal karyotypes in

**Table 1**

Alphabetical list of investigated *Ranunculus* taxa. Voucher: referring to the herbarium specimens deposited in Z+ZT, N: number of investigated individuals per population for chromosome numbers, 2n: chromosome number, KT: number of metaphases per population analysed for karyotype.

<i>Ranunculus</i>	Voucher	N	2n	KT
<i>acris</i> ssp. <i>borealis</i>	14102	6	14	1
<i>acris</i> ssp. <i>borealis</i>	14178	5	14	1
<i>acris</i> ssp. <i>friesianus</i>	13738	6	14	2
<i>aduncus</i>	13567	6	16	3
<i>aduncus</i>	13578	6	16	1
<i>amplexicaulis</i>	11292	5	16	1
<i>arvensis</i>	11909	– <sup>e</sup>	32 <sup>e</sup>	1
<i>brevifolius</i> ssp. <i>brevifolius</i>	11948	– <sup>e</sup>	16 <sup>e</sup>	3
<i>brevifolius</i> ssp. <i>brevifolius</i>	12854	– <sup>f</sup>	16 <sup>f</sup>	1
<i>brevifolius</i> ssp. <i>spindicus</i>	11305	16	16	6
<i>breyneus</i>	12863	9	16	1
<i>bulbosus</i> ssp. <i>aleae</i>	11314	15	16	1
<i>bulbosus</i> ssp. <i>bulbosus</i>	13736	6	16	3
<i>carinthiacus</i>	11468	– <sup>b</sup>	16 <sup>b</sup>	1
<i>carinthiacus</i>	12875	3	16	3
<i>carinthiacus</i>	13568	7	16	2
<i>concinatus</i>	10865	– <sup>b</sup>	16 <sup>b</sup>	3
<i>concinatus</i>	11306	11	32	1
<i>constantinopolitanus</i>	12389	– <sup>c</sup>	28 <sup>c</sup>	1
<i>cortusifolius</i>	14464	4	16	1
<i>damascenus</i>	13867	11	32	2
<i>flammula</i>	11251	15	32	1
<i>glacialis</i>	12556	6	16	1
<i>glacialis</i>	14133	6	16	2
<i>glacialis</i>	13407	7	16	2
<i>gracilis</i>	12193	5	16	8
<i>gramineus</i>	11298	15	16	1
<i>hybridus</i>	31454	15	16	2
<i>hybridus</i>	12109	7	16	1
<i>hybridus</i>	12664	9	16	2
<i>hybridus</i>	12670	9	16	1
<i>isthmicus</i>	14629	6	16	2
<i>lanuginosus</i>	11483	– <sup>b</sup>	28 <sup>b</sup>	3
<i>lanuginosus</i>	11246	15	28	2
<i>lanuginosus</i>	12656	4	28	2
<i>marginatus</i>	11236	11	16	6
<i>marschlinii</i>	11250	15	16	8
<i>millefoliatum</i>	11227	5	16	10
<i>memorosus</i>	11163	– <sup>a</sup>	16 <sup>a</sup>	7
<i>memorosus</i>	33838	– <sup>a</sup>	16 <sup>a</sup>	1
<i>memorosus</i>	10497	– <sup>a</sup>	16 <sup>a</sup>	1
<i>memorosus</i>	11169	– <sup>a</sup>	16 <sup>a</sup>	22
<i>paludosus</i>	13593a	9	16	1
<i>paludosus</i>	13813	– <sup>h</sup>	32 <sup>h</sup>	2
<i>parnassifolius</i> ssp. <i>heterocarpus</i>	13385	8	32	1
<i>platanifolius</i>	11460	– <sup>b</sup>	16 <sup>b</sup>	1
<i>platanifolius</i>	13761	5	16	1
<i>pollinensis</i>	13264	2	16	1
<i>polyanthemos</i>	13734	9	16	2
<i>psilostachys</i>	11316	6	16	1
<i>psilostachys</i>	11422	6	16	8
<i>repens</i>	11547	– <sup>d</sup>	32 <sup>d</sup>	1
<i>repens</i>	11709	– <sup>b</sup>	32 <sup>b</sup>	1
<i>repens</i>	12949	– <sup>g</sup>	32 <sup>g</sup>	2
<i>sartorianus</i>	11443	– <sup>b</sup>	32 <sup>b</sup>	1
<i>sartorianus</i>	12794	4	16	2
<i>scleratus</i>	13481	6	32	1
<i>seguieri</i>	12056	6	16	1
<i>serbicus</i>	11410	10	28	6
<i>thora</i>	11442	– <sup>b</sup>	16 <sup>b</sup>	4
<i>thora</i>	11471	– <sup>b</sup>	16 <sup>b</sup>	2
<i>thora</i>	11417	9	16	1
<i>thora</i>	12033	3	16	1
<i>thora</i>	12111a	9	16	1
<i>thora</i>	12862	15	16	1
<i>velutinus</i>	11678	11	14	4

Chromosome numbers published earlier:

<sup>a</sup> Baltisberger and Huber (1987).

<sup>b</sup> Baltisberger (1990b).

<sup>c</sup> Baltisberger (1991a).

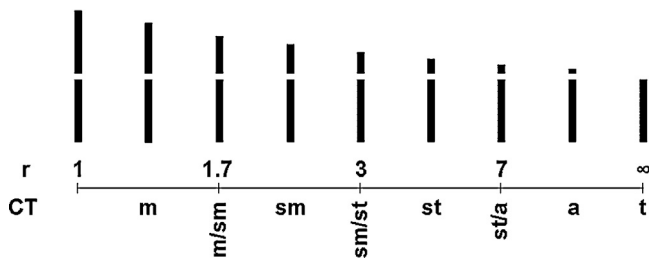
<sup>d</sup> Baltisberger (1991b).

<sup>e</sup> Baltisberger and Baltisberger (1995).

<sup>f</sup> Baltisberger et al. (1996).

<sup>g</sup> Baltisberger (2002).

<sup>h</sup> Baltisberger and Widmer (2004).



**Fig. 1.** Chromosome types (CT) according to Levan et al. (1964). arm ratio ( $r$ ): m = metacentric, arm ratio from 1 to 1.7; sm = submetacentric, arm ratio from 1.7 to 3; st = subtelocentric, arm ratio from 3 to 7; a = acrocentric, arm ratio more than 7; t = telocentric, only one arm. m/sm, sm/st and st/a respectively correspond to the defined borders of the chromosome types. Note that the short arm of an acrocentric chromosome can be very short and easily be interpreted as satellites or even overlooked. The schematic chromosomes given for every chromosome type are also used in Fig. 4 to visualise the karyotypes of the respective clades.

Agave, see Vosa, 2005; Weiss-Schneeweiss and Schneeweiss, 2013). Within *Ranunculus* the chromosomes are rather large but in plants with higher ploidy levels they are generally smaller than in diploids. As the size of chromosomes varies and no correlations could be found we do not consider the size of chromosomes for our analysis.

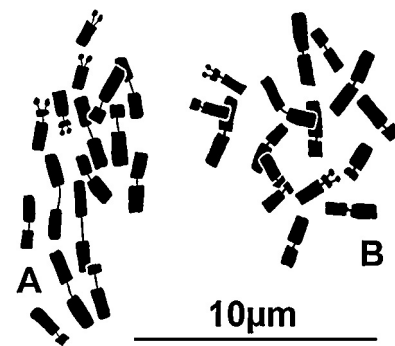
#### 2.4. Karyotypes

To investigate karyotypes, metaphases with well spread chromosomes were drawn with a camera lucida. The nomenclature for chromosome morphology followed Levan et al. (1964). The chromosomes were named according to the position of the centromeres. This is expressed with the ratio “long arm to short arm”. We used the following terms: metacentric (arm ratio 1.0–1.7), submetacentric (ratio 1.7–3.0), subtelocentric (ratio 3.0–7.0), acrocentric (ratio more than 7.0), and telocentric (chromosome only with one arm) (Fig. 1). In diploid plants the chromosomes could be grouped pairwise (in tetraploid plants, in groups with four chromosomes each); in case when the respective chromosomes could not unambiguously be arranged pairwise, the chromosomes with similar size and morphology were lumped in groups not to produce artificial differences (Patau, 1960, 1965; Pohlmann, 1978). Polyploid taxa occur in several clades within *Ranunculus*; to easily compare the respective karyotypes we always indicated the haploid karyotype. To reflect the relationships according to the phylogenetic tree the taxa were grouped according to the clades of the tree, presented in Emadzade et al. (2011). Cluster III is monophyletic (clade III) only after addition of morphological characters (Hörndl and Emadzade, 2012).

Additionally to our own observations on 36 *Ranunculus* species, reliable literature records of karyotypes were collected for further 78 species and assigned to eight main categories within the genus; this dataset included also many earlier studies of the authors (see text). To reconstruct the ancestral karyotypes for the genus *Ranunculus* as a whole, data from the literature (mainly from Goepfert, 1974) on related genera (*Ceratocephala*, *Coptidium*, *Ficaria*, *Halerpestes*, *Kumlienia*, *Trautvetteria*) were also included, this way adding two further karyotypes. Since species of these six genera were not investigated here, these karyotypes are not discussed in detail here but presented briefly after the karyotypes of the *Ranunculus*-species. A comprehensive discussion of these karyotypes can be found in Goepfert (1974).

#### 2.5. Comparing karyotypes from different sources

The borders defining the chromosome types by different arm ratios (viz. meta-, submeta-, subtelo-, and acrocentric; Levan et al., 1964) are pragmatic and also arbitrary. Arm ratios of a given chromosome can be in the range of these borders and with little



**Fig. 2.** Metaphases of *Ranunculus brevifolius* with  $2n = 2x = 16$ . (A) Haploid karyotype with 4 meta- and 3 submeta- to subtelocentric chromosomes (one with satellites) and 1 satellited telocentric chromosome (from Baltisberger, 1990a). (B) Haploid karyotype with 4 meta- and 4 submeta- to subtelocentric chromosomes (one with satellites) (11305).

variation be on one or the other side of the border. This may lead to “different” chromosome types within different metaphases for the same taxon or even the same plant. We therefore do not separate such chromosomes into different types but lump the types for these chromosomes and name them e.g. submeta- to subtelocentric.

A group of chromosomes in a given karyotype may represent a gradient concerning the chromosome indices. Such chromosomes often cannot be sorted in clear pairs. We therefore form a group of these chromosomes. In addition karyotypes can be presented in different modes (different definitions of arm ratio, e.g. “short arm to long arm” or “long arm to total length of the chromosome”) or different borders defining the respective types of chromosomes (e.g. Goepfert [1974] defining metacentric with 1–1.34 [instead of 1–1.7] and submetacentric with 1.34–1.99 [instead of 1.7–3]). This has to be taken in serious consideration in general and especially when comparing karyotypes from different sources in literature.

Another problem is the presence or absence of satellites. Satellites are appendages on the end of a chromosome. They mostly are rather small and often split into two separated bodies which even probably have different distances to the chromosome arm they belong to. Being very small and variously positioned relative to the respective chromosome, satellites may not be visible, easily overlooked or not be interpreted as satellites. Therefore it can be difficult to verify the presence of satellites even in karyologically well known taxa where satellites can be expected (e.g. Baltisberger, 1980 in *Ranunculus polyanthemos* s.l. or Baltisberger, 2002 in *Achillea*). If satellites are positioned on a chromosome with two arms, the presence or absence of satellites is not so important for establishing a karyotype, the set of chromosome types is not influenced and therefore the respective karyotype is not dependent on the visibility of satellites. But, if satellites are positioned on a telocentric chromosome (with only one arm) the satellites get much more importance for the characterization of the karyotype. If the satellites are not visible the chromosome will be interpreted as telocentric. If visible, the satellites can be interpreted as a very short arm (the respective chromosome than mostly being acrocentric) or as satellites (and the respective chromosome than named as satellited telocentric) which has an impact on the karyotype formula. This may be illustrated with *Ranunculus brevifolius*. The smallest chromosome of the haploid set can be indicated in different ways depending on the interpretation of the satellites: as a telocentric chromosome with satellites (Fig. 2A) or as a subtelocentric chromosome without satellites (Fig. 2B). The visibility of the satellites of the other small chromosome (in Fig. 2 in both metaphases with satellites) does not influence the karyotype as it is in both cases a subtelocentric chromosome (with or without satellites). To be on the safe side, we do not mention satellites in the characterization

of karyotypes and interpret satellites on telocentric chromosomes as a very short arm (see e.g. comments in clade VIII and IX).

### 2.6. Reconstruction of karyotype evolution

Altogether our dataset comprised karyotypes for 123 species. We optimized the ten major karyotypes (eight in *Ranunculus*, two in other genera; see text) as character states onto the molecular tree topology of Emadzade et al. (2011), including the genera of Ranunculaceae, by using Mesquite 3.02 using parsimony to reconstruct ancestral states (Maddison and Maddison, 2015); Fig. 5. We preferred this tree topology based on molecular data only over the total evidence tree in Hörandl and Emadzade (2012), because the latter analysis already included some karyological characters.

## 3. Results

The taxa are arranged according to the clades in Emadzade et al. (2011) and alphabetically within a clade. The results are presented and completed with the indications in literature. The evolution of karyotypes as well as more general aspects are discussed in final parts.

### 3.1. Chromosome numbers

There are two basic chromosome numbers within the genus *Ranunculus* viz.  $x=7$  (only in clade VI) and the common  $x=8$  (all other clades). The chromosome numbers counted are compiled in Table 1. The chromosome numbers of *Ranunculus concinnatus*, *R. isthmicus* and *R. marschlinii* were not known up to now; the numbers found in other taxa confirmed the indications in literature.

### 3.2. Karyotypes

The number of analyzed metaphases per population is given in column KT in Table 1. To illustrate the main categories of karyotypes a metaphase of one species representative for each clade is presented in Fig. 3. Literature was checked for karyotype information for all taxa indicated in the phylogenetic tree. The karyotypes newly described in this article and those from literature are compared and discussed. Searching in literature for karyotype information it turned out that papers with figures of good metaphases were very helpful. We therefore offer pictures of one metaphase of each investigated taxon in the electronic Appendix 2.

### 3.3. Clade I

Clade I corresponds to section *Thora*, comprising all three European species of this section, viz. *Ranunculus brevifolius* Ten., *R. hybridus* Biria and *R. thora* L. They all grow in mostly stony habitats in the mountains of central and southern Europe. *Ranunculus hybridus* and *R. thora* grow in the southern parts of the Alps and a bit southwards while *R. brevifolius* is restricted to more southern mountains in Italy and the Balkan Peninsula. Plants from central Italy as well as Albania and Montenegro are named *R. brevifolius* ssp. *brevifolius*. The plants from Greece (including Crete) differ in having more numerous and more deeply lobed leaves and smaller achenes. They are named *R. brevifolius* ssp. *pindicus* (Hausskn.) E.Mayer. The differences between the two subspecies are weak and probably not consistent but the subspecies show a clear disjunct geographic distribution.

All taxa are diploid with  $2n=2x=16$  chromosomes. We investigated all three species of this clade, and all plants showed the same haploid karyotype with 4 meta- and 4 submeta- to subtelocentric chromosomes (see Fig. 3A), mostly with satellites on a small

subtelocentric chromosome. This corresponds to karyotype indications in the literature for *R. brevifolius* (Baltisberger, 1990a), *R. hybridus* (Goepfert, 1974) and *R. thora* (Goepfert, 1974; Diosdado and Pastor, 1993a). D'Ovidio et al. (1986) give the chromosome number of *R. thora* (no karyotype) but checking their figure with the chromosomes of *R. thora*, the karyotype appears to be the same.

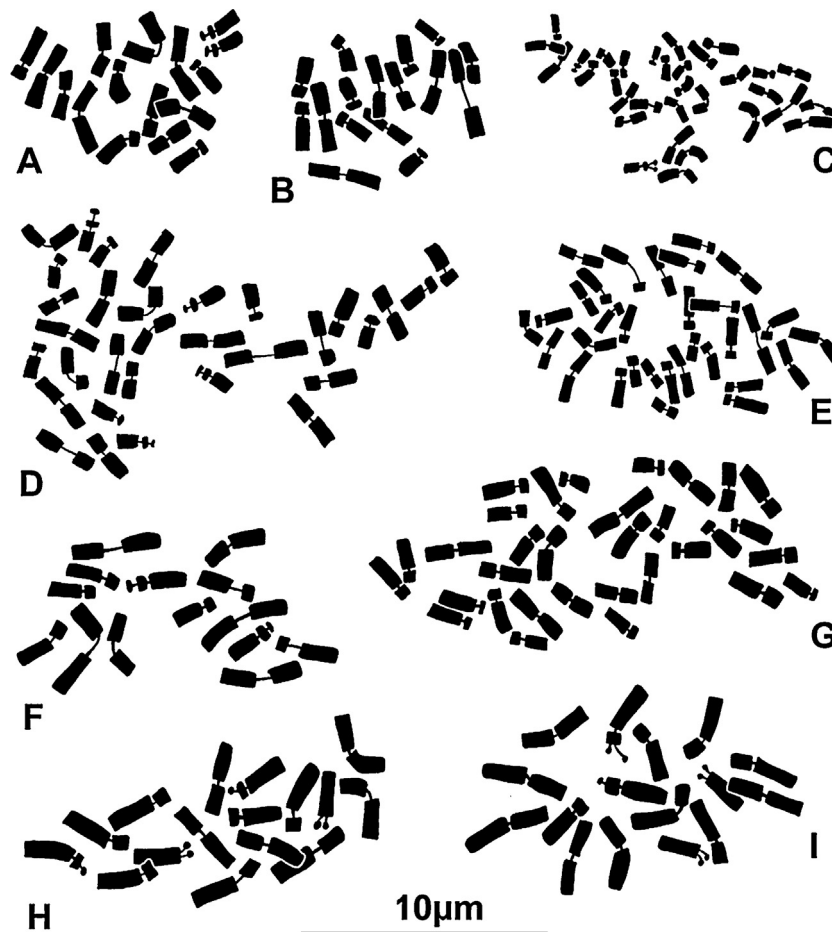
### 3.4. Clade II

The 17 species mentioned in clade II can be circumscribed as “white-flowered European alpinines”. Nevertheless one species (*Ranunculus gramineus* L.) has yellow flowers. The species within this clade are morphologically classified in four sections (Hörandl and Emadzade, 2010) and mostly grow in mountains of central and southern Europe, *Ranunculus glacialis* L. also occurs in European arctic and subarctic areas (including Greenland), while *R. camissonis* Aucl. (closely related to *R. glacialis*) is restricted to the Beringian region (Alaska as well as adjacent parts of Asia).

*Ranunculus kuepferi* W.Greuter & Burdet (Huber, 1988), *R. parnassifolius* L. (Bueno Sanchez et al., 1992) and *R. seguieri* Vill. (Baltisberger, 1992) consist of diploid and polyploid subspecies but most of the taxa in clade II are diploid with  $2n=16$  chromosomes. We investigated six species of this clade: the diploid *R. amplexicaulis* L., *R. glacialis*, *R. gramineus*, *R. platanifolius* L., and *R. seguieri* as well as the tetraploid *R. parnassifolius* ssp. *heterocarpus* Künfer. All species showed the same haploid karyotype with 4 meta- and 4 submeta- to subtelocentric chromosomes and (see Fig. 3B), often with satellites on a small subtelocentric chromosome.

Similar karyotype informations are given in literature for 16 of the 17 taxa mentioned in clade II (no cytological data are available for *Ranunculus camissonis*): *R. acetosellifolius* Boiss. (Diosdado and Pastor, 1993a), *R. aconitifolius* L. (Goepfert, 1974; Diosdado and Pastor, 1993a), *R. alpestris* L. (Bauer, 1954; Goepfert, 1974; Baltisberger and Müller, 1981; Marchi and Visona, 1982; Diosdado and Pastor, 1993a; Baltisberger et al., 1996; Baltisberger and Widmer, 2009a [including *R. alpestris* ssp. *leroyi* Lainz]); *R. amplexicaulis* (Künfer, 1974; Diosdado and Pastor, 1991a), *R. bilobus* Bertol. (Müller and Baltisberger, 1984; Baltisberger et al., 1996; Baltisberger and Widmer, 2009a), *R. cacuminis* Strid & Papan. (Baltisberger, 1994; Baltisberger and Widmer, 2009a), *R. crenatus* Waldst. & Kit. (Goepfert, 1974; Sopova and Sekovski, 1982; Müller and Baltisberger, 1984; Baltisberger, 2006; Baltisberger and Widmer, 2009a), *R. glacialis* (Bauer, 1954; Goepfert, 1974; Diosdado and Pastor, 1993a), *R. gramineus* (Kurita, 1957; Goepfert, 1974; Künfer, 1974; Marchi and Visona, 1982; Diosdado and Pastor, 1991a), *R. kuepferi* (Künfer, 1974), *R. magellensis* Ten. (Gregson, 1965; Müller and Baltisberger, 1984; Baltisberger et al., 1996; Baltisberger and Widmer, 2009a), *R. parnassifolius* (Goepfert, 1974; Künfer, 1974; Diosdado and Pastor, 1991a), *R. platanifolius* (Bauer, 1954; Marchi and Visona, 1982; Sopova and Sekovski, 1982; Diosdado and Pastor, 1993a), *R. pyrenaicus* L. (Künfer, 1974; Diosdado and Pastor, 1991a), *R. seguieri* (Künfer, 1974; Baltisberger and Müller, 1981; Baltisberger, 1990a, 1990b [on pentaploid *R. seguieri* ssp. *montenegrinus* (Halacsy) Tutin], 1992 [on tetraploid *R. seguieri* ssp. *montenegrinus*]; Diosdado and Pastor, 1993a), *R. traunfellneri* Hoppe (Goepfert, 1974; Müller and Baltisberger, 1984; Baltisberger and Widmer, 2009a).

The following sources give the chromosome number but no karyotype for the respective taxon, but checking figures of the metaphases, the following karyotypes seem to be the same as given in the references listed in the previous paragraph: *R. gramineus* (Marchi and Visona, 1982), *R. platanifolius* (D'Ovidio et al., 1986), *R. parnassifolius* and the closely related *R. wettsteinii* Dörfel. (Baltisberger, 1992) the latter probably of allopolyploid origin (Cires et al., 2014).



**Fig. 3.** Metaphases of *Ranunculus* species. (A) *R. thora* ( $2n=2x=16$ , 12862; clade I). (B) *R. glacialis* ( $2n=2x=16$ , 14133; clade II). (C) *R. sceleratus* ( $2n=4x=32$ , 13481; cluster III). (D) *R. auricomus* ( $2n=4x=32$ ; clade IV; from Baltisberger, 2006). (E) *R. flammula* ( $2n=4x=32$ , 11251; clade V). (F) *R. acris* (ssp. *borealis*;  $2n=2x=14$ , 14178; clade VI). (G) *R. arvensis* ( $2n=4x=32$ , 11909; clade VII). (H) *R. bulbosus* (ssp. *aleae*;  $2n=2x=16$ , 11314; clade VIII). (I) *R. psilostachys* ( $2n=2x=16$ , 11422; clade IX).

Kurita (1957) indicates a haploid karyotype for *R. aconitifolius* with the basic chromosome number  $x=7$  fitting with that of clade VI. This completely differs from all other species of clade II. The plants investigated did not originate from a natural habitat but from a botanical garden (Milano, Italy). A confusion of labels seems rather likely (therefore not mapped onto the tree).

### 3.5. Cluster III

Cluster III comprises the white-flowered aquatic *Ranunculus* taxa (sect. *Batrachium*) as well as yellow-flowered wetland species (sect. *Hecatonia*) and *Ranunculus* taxa from mountains of New Zealand (sect. *Pseudadonis*). This morphologically very heterogeneous, worldwide distributed group has a complex reticulate evolutionary history and was rendered monophyletic after inclusion of morphological characters (Hörndl, 2014). Therefore, we treat the group here as the other clades of the genus. Karyologically, the group is quite homogeneous and shows the common basic chromosome number  $x=8$ . Many polyploids are known. We investigated *Ranunculus sceleratus* L. which is a mostly tetraploid and sometimes polyploid, eurasiatic, widespread and yellow-flowered species from wetlands. The investigated plants proved to be tetraploid and showed a haploid karyotype with 4 metacentric and 4 submetacentric to subtelocentric chromosomes (see Fig. 3C). The satellites on one of the small subtelocentric chromosomes of the haploid set were not always visible. Similar karyotypes for *R. sceleratus* are given by Goepfert (1974), Kapoor (1981), D'Ovidio et al. (1986) and Vaidya and Joshi (2003).

With 1 meta-, 1 submetacentric and 6 subtelocentric chromosomes a clearly deviating karyotype is given by Sopova and Sekovski (1982) on diploid plants from Macedonia; we cannot say if *R. sceleratus* really shows great variability concerning both ploidy level and karyotype or if this information is based on misidentified plants. Another deviating indication for the same species with 5 metacentric and 3 submetacentric to subtelocentric chromosomes is presented in Liao et al. (1991) and Yang (2001) on Chinese material.

Due to small differences in defining the chromosome types (e.g. ranges of arm ratio for metacentric 1–1.34 instead of 1–1.7 and submetacentric 1.34–1.99 instead of 1.7–3) Goepfert (1974) indicates more variation of karyotypes within the species of clade III. Applying the definitions of chromosome types according to Levan et al. (1964) on the data of Goepfert (1974) he got karyotypes similar to the ones we found. This is the case for *R. gmelinii* DC., another yellow-flowered wetland species mentioned in clade III as well as for the New Zealand taxa and the aquatic taxa investigated by Goepfert (1974, see below). The alpine New Zealand species are highly polyploid (Rendle and Murray, 1989). The same karyotype is indicated for *R. insignis* Hook.f. by Goepfert (1974) as well as for *R. lyallii* Hook.f. by Gregson (1965) and Goepfert (1974). No karyotypes are given by Hair (1983) but checking the figure of the metaphase of *R. insignis* the karyotype seems to be quite similar. The karyotypes of the aquatic species of *Ranunculus* (sect. *Batrachium*) are rather uniform and constant and similar to what we found in *R. sceleratus* (e.g. *R. peltatus* Moench, *R. penicillatus* [Dum.] Bab., *R. sphaerospermus* Boiss. & Blanche, and *R. trichophyllus* Chaix; Cook, 1966; Goepfert, 1974; Dahlgren, 1991; Diosdado et al., 1993).

### 3.6. Clade IV

For this study we did not investigate any species of clade IV here, all information originates from literature. The species within this clade show the common basic chromosome number  $x=8$ , they mostly are perennial and have yellow flowers. Clade IV is classified as *Ranunculus* sect. *Auricomus* and includes species from North American and Asian mountains and the Arctic as well as the Eurasian lowland *R. auricomus* complex (Emadzade et al., 2015). Many polyploids exist, and especially within the highly variable complex *R. auricomus* hundreds of agamospermic taxa are known, but only diploid sexual species (*R. carpaticola*, *R. cassubicifolius*, and *R. notabilis*) were included in the phylogeny here. The karyotype consists of 4 metacentric and 4 submeta- to subtelocentric chromosomes (see Fig. 3D), satellites are often indicated for one of the small subtelocentric chromosomes. A similar karyotype is given (or at least can be seen in the respective figures) for various taxa of this clade (e.g. *R. abortivus* L., *R. brotherusii* Freyn, *R. cardiophyllus* Hook., *R. cassubicifolius* W.Koch, *R. eschscholtzii* Schlecht., *R. nivalis* L., *R. notabilis* Hörandl & Guterm., *R. rhomboideus* Goldie) in Kurita (1957), Kurosawa (1971), Goepfert (1974), Marchi and Visona (1982), Zhuravleva and Malakhova (1983), Masci et al. (1987), Vuillemin (1990), Liao et al. (1991), Hörandl et al. (1997), Yang (2001) and Baltisberger (2006).

### 3.7. Clade V

Clade V comprises yellow-flowered semiaquatic or terrestrial wetland species with undivided leaves (sect. *Flammula*). The taxa show the basic chromosome number  $x=8$ , and diploids as well as polyploids are known. The widespread *Ranunculus flammula* L. is tetraploid as were the investigated plants. They showed a haploid karyotype of 4 metacentric and 4 submeta- to subtelocentric chromosomes (see Fig. 3E). A similar karyotype for *R. flammula* is given in Gregson (1965), Goepfert (1974), Diosdado and Pastor (1991b), and Peruzzi and Cesca (2002). Marchi and Visona (1982) present a figure of a metaphase of *R. flammula* where the karyotype seems to be the same. The same karyotype is indicated for *R. ficariifolius* Leveille & Vaniot (Yang, 2001).

It is interesting that several sources indicate a deviating karyotype (chromosomes: 3 metacentric, 4 submetacentric and 1 subtelo- to acrocentric) for two species of clade V viz. *R. lateriflorus* DC. (Gregson, 1965; Hindakova, 1965; Goepfert, 1974; Galland, 1988; Diosdado and Pastor, 1991b), and *R. ophioglossifolius* Vill. (Gregson, 1965; Goepfert, 1974; Capineri et al., 1978; Diosdado and Pastor, 1991b). We accept this as a separate character state in Fig. 4 (Va) and Fig. 5.

### 3.8. Clade VI

The taxa of this clade VI are mostly perennial and occur in Eurasia, North America, and Africa. They have (as many other species) yellow flowers. The unique character within this clade is the basic chromosome number  $x=7$  (instead of  $x=8$  as all other taxa of *Ranunculus*) which was also an important argument to classify the clade as a section (sect. *Ranunculus*; Hörandl et al. 2012). Diploid and polyploid species are known. We investigated five species viz. the two diploid species *Ranunculus acris* L. (the two subspecies ssp. *borealis* [Regel] Nyman and ssp. *friesianus* [Jord.] Rouy & Fouc.) and *R. velutinus* Schur as well as the three tetraploid species *R. constantinopolitanus* (DC.) d'Urv., *R. lanuginosus* L., and *R. serbicus* Vis. All showed the same haploid karyotype of 3 metacentric and 4 submeta- to subtelocentric chromosomes (see Fig. 3F) mostly with satellites on one of the subtelocentric chromosomes.

Many indications of similar karyotypes for several species mentioned in the phylogenetic tree in clade VI are given in literature:

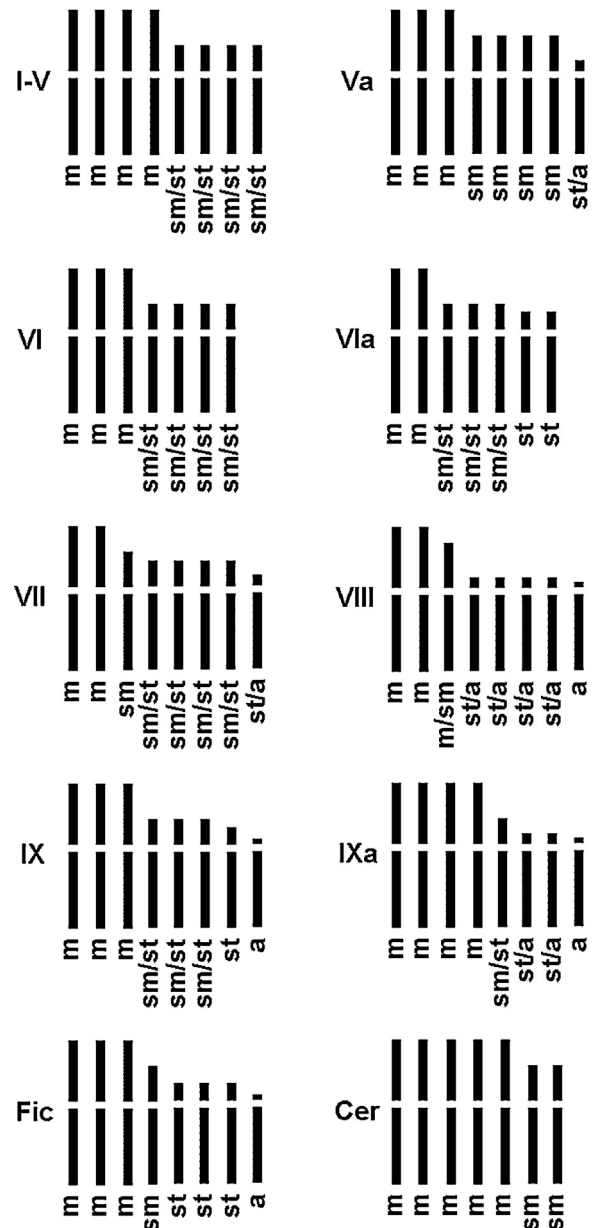
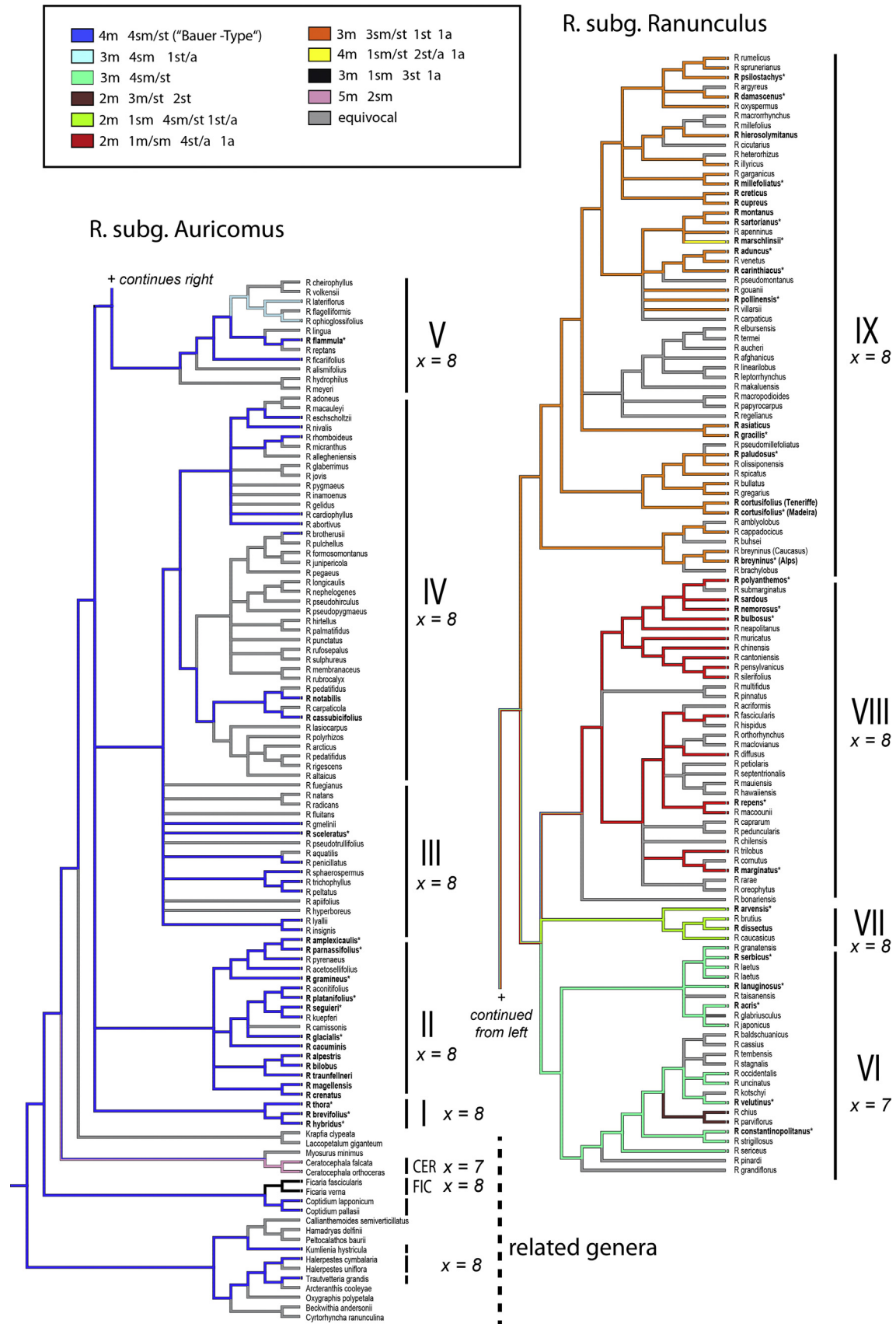


Fig. 4. Character states of karyotypes of *Ranunculus* and related genera. The indicated chromosome types correspond to the descriptions in the text. To visualise the karyotypes the schematic chromosomes correspond to Fig. 1. The Roman numerals (I–IX) correspond to the numbers of the clades; Va (designates two species of clade V): *R. lateriflorus* and *R. ophioglossifolius*; VIa: *R. chius* and *R. parviflorus*; IXa: *R. marschlinii*; Fic: *Ficaria*; Cer: *Ceratocephala*.

*R. acris* (Kurita, 1957; Gregson, 1965; Goepfert, 1974; Marchi et al., 1975; Kapoor, 1981; Sopova and Sekovski, 1982; Zhuravleva and Malakhova, 1983; Diosdado and Pastor, 1992; Baltisberger and Baltisberger, 1995; Baltisberger, 2006; Baltisberger and Widmer, 2009b), *R. constantinopolitanus* (Kurita, 1957; Goepfert, 1974), *R. granatensis* Boiss. (Goepfert, 1974; Diosdado and Pastor, 1992), *R. japonicus* Thunb. (Kurita, 1957; Liao and Chen, 1991; Liao et al., 1991, 1996; Yang et al., 1994; Yang, 2001), *R. laetus* Wallich ex D. Don (Yang, 2001), *R. lanuginosus* (Goepfert, 1974; Marchi et al., 1975; Agapova, 1981; Sopova and Sekovski, 1982), *R. occidentalis* Nutt. (Goepfert, 1974), *R. serbicus* (Goepfert, 1974; Marchi et al., 1975; Sopova and Sekovski, 1982; Baltisberger and Baltisberger, 1995; Baltisberger, 2002, 2006), *R. sericeus* Banks & Solander (Goepfert, 1974), *R. strigillosus* Boiss. & Hutt. (Goepfert, 1974), *R.*



**Fig. 5.** Reconstruction of karyotype evolution on the molecular tree topology of *Ranunculus* and related genera (after Emadzade et al., 2011). The character states correspond with the ten major karyotypes shown in Fig. 4. The major clades within *Ranunculus* are indicated by Roman numerals (cluster III is monophyletic with inclusion of morphological characters, Hörndl & Emadzade 2012). Species in bold represent own observations (with asterisk = presented here, without asterisk = from previous papers of the authors); all other records are from the literature (see text).

*uncinatus* D. Don (Goepfert, 1974), and *R. velutinus* (Goepfert, 1974; Marchi et al., 1975; Sopova and Sekovski, 1982).

Checking figures of metaphases of the respective species the following sources (without indicating the karyotype) show the same haploid karyotype of 3 metacentric and 4 submeta- to subtelocentric chromosomes: *R. acris* (Marchi and Visona, 1982; Krasnikova et al., 1983; Lavrenko and Serditov, 1985), *R. lanuginosus* (D'Ovidio et al., 1986; Baltisberger et al., 1996), *R. velutinus* (D'Ovidio et al., 1986). Additionally, it can be expected that the tetraploid *R. fibrillosus* K. Koch (not included in the phylogenetic tree of Hörandl and Emadzade, 2012) also is a member of Clade VI as it is closely related to *R. constantinopolitanus* (Davis, 1960, 1965). This is supported by the cytological data as *R. fibrillosus* has the basic chromosome number  $x = 7$ , and shows the same karyotype (Baltisberger, 1991b).

A slightly deviating karyotype with 2 metacentric, 3 submeta- to subtelocentric and 2 subtelocentric chromosomes is indicated for *R. chius* DC. (Goepfert, 1974) and *R. parviflorus* L. (Goepfert, 1974; D'Ovidio et al., 1986; Diosdado and Pastor, 1993b). We accept this as a separate character state in Fig. 4 (VIa) and Fig. 5.

Two further sources indicate a haploid karyotype with the basic chromosome number  $x = 8$  which completely differs from all other species of clade VI: *R. constantinopolitanus* with  $2n = 4x = 32$  (Agapova, 1981) and *R. laetus* with  $2n = 2x = 16$  (Vaidya and Joshi, 2003). Such a high variability concerning the chromosomes is surprising. However, more likely the identification of these plants is questionable, and therefore we did not map these karyotypes onto the tree.

### 3.9. Clade VII

All species in clade VII show the common basic chromosome number  $x = 8$ . Although the clade is rather small it is nevertheless divided into two distinct groups. A monotypic branch (sect. *Arvensis*) consists of the annual tetraploid *Ranunculus arvensis* L. with spiny achenes. The other group forms a clade, is morphologically very heterogeneous and comprises 3–5 species (sect. *Trisecti*). We investigated *R. arvensis*. The plants proved to be tetraploid and showed a karyotype of 2 meta-, 1 submeta- and 4 submeta- to subtelocentric chromosomes and 1 subtelo- to acrocentric chromosome (see Fig. 3G). The same karyotype is indicated for *R. arvensis* (Goepfert, 1974; Sopova and Sekovski, 1982; D'Ovidio et al., 1986; Diosdado and Pastor, 1993b) as well as the taxa of the other group mentioned in clade VII viz. *R. brutius* Ten. (D'Ovidio et al., 1986), *R. caucasicus* M. Bieb. (Agapova, 1981) and *R. dissectus* M. Bieb. (Agapova, 1981; Baltisberger and Tan, 1991).

### 3.10. Clade VIII

The taxa of clade VIII are mostly perennial and occur worldwide. They have (as many other species) yellow flowers. They differ from clade VI in the basic chromosome number  $x = 8$ , and were separately classified as sect. *Polyanthes*. Both diploid and polyploid species are known. We investigated five species viz. the diploid *Ranunculus bulbosus* L. (the two subspecies ssp. *aleae* [Willk.] Rouy & Fouc. and ssp. *bulbosus*), *R. marginatus* d'Urv., *R. nemorosus* DC. (= *R. serpens* Schrank ssp. *nemorosus* [DC.] G. Lopez, = *R. tuberosus* Lapeyr.) and *R. polyanthemos* L. as well as the tetraploid *R. repens* L. All showed the same haploid karyotype with 2 metacentric, 1 meta- to submeta-centric, 4 subtelo- to acrocentric chromosomes and 1 acrocentric chromosome (see Fig. 3H). For one of the subtelo- to acrocentric chromosomes satellites are often indicated. We consider the smallest chromosome as acrocentric but in the literature the interpretation of this chromosome is not unanimous: it is interpreted as telocentric with satellites (e.g. Baltisberger, 1980; Jasinska, 1980) or as acrocentric with a rather short arm and without satellites (Goepfert, 1974) (see also comment under clade IX).

Similar karyotypes are given in literature for many species mentioned in clade VIII: *R. bulbosus* (Gregson, 1965; Goepfert, 1974; Marchi et al., 1975; Kapoor, 1981; Sopova and Sekovski, 1982; Agapova, 1983; Diosdado and Pastor, 1992), *R. cantoniensis* DC. (Yang et al., 1994; Fujishima et al., 1995; Liao et al., 1995), *R. chinensis* Bunge (Goepfert, 1974; Fujishima et al., 1995; Liao et al., 1995; Yang and Zhou, 1999; Yang, 2001), *R. diffusus* DC. (Liao et al., 1996; Vaidya and Joshi, 2003), *R. fascicularis* Muhl. (Goepfert, 1974), *R. macounii* Britton (Goepfert, 1974), *R. marginatus* (Gregson, 1965; Goepfert, 1974; Lentini et al., 1988; Baltisberger and Baltisberger, 1995), *R. muricatus* L. (Gregson, 1965; Goepfert, 1974; Subramanian, 1985; Diosdado and Pastor, 1993b), *R. neapolitanus* Ten. (Gregson, 1965; Goepfert, 1974; Agapova, 1981; Sopova and Sekovski, 1982), *R. nemorosus* (Goepfert, 1974; Baltisberger, 1980; Jasinska, 1980; Agapova, 1981; Sopova and Sekovski, 1981; Diosdado and Pastor, 1992), *R. pennsylvanicus* L.f. (Gregson, 1965; Goepfert, 1974), *R. polyanthemos* (Goepfert, 1974; Koeva-Todorovska and Nenova, 1976/77; Baltisberger, 1980; Agapova and Zemskova, 1983; Jasinska, 1980; Sopova and Sekovski, 1981, 1982; Zhuravleva and Malakhova, 1983), *R. repens* (Gregson, 1965; Goepfert, 1974; Marchi et al., 1975; Agapova, 1981; Kapoor, 1981; Sopova and Sekovski, 1982; Zhuravleva and Malakhova, 1983; Diosdado and Pastor, 1992), *R. sardous* Crantz (Gregson, 1965; Goepfert, 1974; Capineri et al., 1978; Sopova and Sekovski, 1982; Fujishima, 1990; Diosdado and Pastor, 1993b; Baltisberger and Widmer, 2009b), *R. silerifolius* Lev. (Liao et al., 1995, 1996), *R. trilobus* Desf. (Gregson, 1965; Goepfert, 1974; Diosdado and Pastor, 1993b).

Additionally similar karyotypes are given or shown in figures for *R. polyanthemoides* Schur, *R. polyanthemophyllus* W. Koch & H.E. Hess, and *R. serpens* Schrank (Baltisberger, 1980) as well as *R. thomasi* Ten. (Baltisberger, 1980; Marchi and Visona, 1982; D'Ovidio et al., 1986), all closely related to species in clade VIII but not included in the phylogenetic tree of Hörandl and Emadzade (2012). No species of the group *R. lappaceus* Smith (growing in Australia and New Zealand) is included in the phylogenetic tree here but these *Ranunculi* nevertheless belong to section *Polyanthes* according to the molecular ITS tree in Hörandl et al. (2005, see also Hörandl and Emadzade, 2012, Appendix 6). They show the same karyotype (including additional secondary constrictions; Briggs, 1962).

Checking figures of metaphases of the respective species the following sources (without indicating the karyotype) show the same chromosome types in the haploid karyotype (2 metacentric, 1 meta- to submetacentric, 4 subtelo- to acrocentric and 1 acrocentric): *R. bulbosus* (Marchi and Visona, 1982), *R. polyanthemos* (Andersson, 1958; Lavrenko and Serditov, 1985), and *R. repens* (Lavrenko and Serditov, 1985).

Several karyotypes are given for *R. silerifolius* by Fujishima (1983, 1988) and Fujishima et al. (1995). Some karyotypes deviate strongly (e.g. with 6 metacentric and 2 subtelo- to acrocentric chromosomes) which is different to all karyotypes of any species indicated in the genus *Ranunculus*. The species belongs to a polymorphic hybrid complex (*R. cantoniensis*-group, Liao et al., 2008; Li et al., 2014). Variation could be due to chromosomal rearrangements after hybridization, but also mis-identification of species cannot be ruled out (Kuo et al., 2005).

### 3.11. Clade IX

All species in clade IX are perennial and have yellow flowers (except *R. asiaticus* L. with yellow, white or red flowers). The basic chromosome number is  $x = 8$ , and diploid and polyploid taxa are known. Clade IX consists of two monophyletic subclades (viz. sections *Oreophili* Tschuradze and *Euromontani* Hörandl) and the rest being a rather big paraphyletic group (sect. *Ranunculastrum* DC.) (Hörandl and Emadzade, 2012). These three units differ in



pubescence of the receptacle, size of the beak of the achenes, underground parts and in ecology. We investigated 14 taxa representing all three groups: *R. breyninus* Crantz (= *R. oreophilus* M.Bieb.) from sect. *Oreophili*, six species of sect. *Euromontani* (*R. aduncus* Gren. & Godr., *R. carinthiacus* Hoppe, *R. concinnatus* Schott [not included in the phylogenetic tree of Hörandl and Emadzade, 2012], *R. marschlinsii* Steud., *R. pollinensis* Chiov., and *R. sartorianus* Boiss. & Heldr.), as well as *R. cortusifolius* Willd., *R. damascenus* Boiss. & Gaill., *R. gracilis* Schleich., *R. isthmicus* Boiss. (not included in the phylogenetic tree of Hörandl and Emadzade, 2012), *R. millefoliatus* Vahl, *R. paludosus* Poir., and *R. psilostachys* Griseb. from sect. *Ranunculastrum*. Except for *R. marschlinsii* (see below) they all showed the same haploid karyotype with 8 chromosomes, 3 metacentric, 3 submeta- to subtelocentric, 1 subtelocentric and 1 acrocentric (Fig. 3I). Satellites were sometimes visible at one of the subtelocentric chromosomes. As the short arm of the acrocentric chromosome is often very short and sometimes split into two bodies this chromosome could also be interpreted as telocentric with satellites (see also comment under clade VIII).

Similar karyotypes are given for many species included in this clade by Hörandl and Emadzade (2012). So for two species of sect. *Oreophili*: *R. cappadocicus* Willd. (Goepfert, 1974; Agapova, 1983) and *R. breyninus* (Goepfert, 1974; Sopova and Sekovski, 1982; Agapova, 1983; Baltisberger and Widmer, 2009b).

For several taxa of the sect. *Euromontani* the same karyotype is indicated: *R. aduncus* (Masci et al., 1987), *R. carinthiacus* (Sopova and Sekovski, 1981; Dickenmann, 1982; Diosdado and Pastor, 1992), *R. gouanii* Willd. (Goepfert, 1974; Diosdado and Pastor, 1992), *R. montanus* Willd. (including indications for *R. montanus* s.l.; Goepfert, 1974; Marchi et al., 1975; Dickenmann, 1982; Sopova and Sekovski, 1982; Baltisberger, 1984, 2002, 2006), *R. sartorianus* (Sopova and Sekovski, 1981), *R. venetus* Huter ex Landolt (Masci et al., 1987), and *R. villarsii* DC. (= *R. grenierianus* Jord.; Goepfert, 1974; Dickenmann, 1982).

Similar karyotypes are also given for many species of sect. *Ranunculastrum*: *R. asiaticus* (Kurita, 1957; Goepfert, 1974; Tzanoudakis, 1986; Tak and Wafai, 1996; Baltisberger and Widmer, 2004, 2005), *R. bullatus* L. (Goepfert, 1974; Ottonello et al., 1985), *R. cortusifolius* (Goepfert, 1974; Dalgaard, 1985; Baltisberger et al., 1990; Baltisberger and Widmer, 2006), *R. creticus* L. (Goepfert, 1974; Tzanoudakis, 1986; Baltisberger and Widmer, 2005), *R. cupreus* Boiss. & Heldr. (Baltisberger and Widmer, 2005), *R. damascenus* (Baltisberger and Widmer, 2004), *R. garganicus* Ten. (probably a synonym of *R. millefoliatus*; Marchi et al., 1975), *R. gracilis* (Popova, 1973), *R. gregarius* Brot. (Goepfert, 1974), *R. hierosolymitanus* Boiss. (Grau, 1989; Baltisberger and Widmer, 2004), *R. illyricus* L. (Popova, 1973; Goepfert, 1974; Sopova and Sekovski, 1982; D'Ovidio et al., 1986), *R. millefoliatus* (Popova, 1973; Goepfert, 1974; Sopova and Sekovski, 1982), *R. ollisiponensis* Pers. (Diosdado and Pastor, 1990), *R. oxyspermus* Willd. (Popova, 1973; Goepfert, 1974; Sopova and Sekovski, 1981), *R. paludosus* Poir. (Marchi et al., 1975; Diosdado and Pastor, 1990), *R. psilostachys* (Popova, 1973; Goepfert, 1974; Sopova and Sekovski, 1982), *R. rumelicus* Griseb. (Popova, 1973; Sopova and Sekovski, 1982), *R. spicatus* Desf. (Diosdado and Pastor, 1990), and *R. sprunerianus* Boiss. (Popova, 1973; Sopova and Sekovski, 1981).

Checking figures of metaphases of the respective species in the following sources (without indicating the karyotype) show the same chromosome types in the haploid karyotype (3 metacentric, 3 submeta- to subtelocentric, 1 subtelocentric and 1 acrocentric): *R. apenninus* (Chiov.) Pignatti (Marchi and Visona, 1982; Masci et al., 1987), *R. breyninus* (Marchi and Visona, 1982; Masci et al., 1987), *R. garganicus* (Marchi and Visona, 1982), *R. illyricus* (Marchi and Visona, 1982), *R. millefoliatus* (Marchi and Visona, 1982), *R. montanus* (Giordani et al., 1980; Masci et al., 1987), and *R. pollinensis* (Marchi and Visona, 1982; Masci et al., 1987).

Additionally a similar karyotype is indicated for *R. ruscinonensis* Landolt (Diosdado and Pastor, 1992), a species belonging to sect. *Euromontani* (but not included in Hörandl and Emadzade, 2012).

A slightly different karyotype with 4 metacentric, 1 submeta- to subtelocentric, 2 subtelocentric to acrocentric and 1 acrocentric chromosomes was found in *R. marschlinsii* (sect. *Euromontani*), an endemic species from Corsica. It cannot be said if this is a generally different karyotype within *R. marschlinsii* or if this is only the case for the single population that was investigated. As even the chromosome number of *R. marschlinsii* was not known up to now no indication concerning karyotype is available in literature. As all 8 analyzed metaphases showed exactly the same karyotype we accept this as a separate character state in Fig. 4 (IXa) and Fig. 5.

### 3.12. Karyotypes of related genera

We searched in literature for data about the karyotypes of the related genera of *Ranunculus*. The karyotype identical to the one found in the clades I–V in *Ranunculus* with 4 meta- and 4 submeta- to subtelocentric chromosomes (Fig. 4: I–V) is indicated for *Coptidium* (Goepfert, 1974; Lavrenko and Serditov, 1985), *Halerpestes* (Kurita, 1957; Goepfert, 1974; Yang, 2001), *Kumlienia* (Goepfert, 1974) and *Trautvetteria* (Kurita, 1957; Goepfert, 1974). Karyotypes different from all the karyotypes within the genus *Ranunculus* are found in *Ficaria* and *Ceratocephala*. The karyotype of *Ficaria* (Fig. 4: Fic) consists of 3 meta-, 1 submeta-, 3 subtelocentric and 1 acrocentric chromosomes (Gregson, 1965; Marchant and Brighton, 1971; Goepfert, 1974; Capineri et al., 1978; Ferrarella et al., 1979; Valdes-Bermejo, 1980; Diosdado and Pastor, 1993c), and *Ceratocephala* with 5 meta- and 2 submetacentric chromosomes (Fig. 4: Cer) shows a karyotype with the basic chromosome number  $x=7$  (Goepfert, 1974).

## 4. Discussion

### 4.1. Karyotype evolution

The mapping of karyotypes as character states onto the molecular tree confirmed a congruence of karyotypes to the tree topology with well-supported clades (Fig. 5). The congruence of the molecular data, where characters were derived from plastid and nuclear sequences, to chromosomal features, suggests that karyotype evolution reflects well the evolutionary history of the genus. The ancestral karyotype of *Ranunculeae*, of the genus *Ranunculus* s.str. and also of the ancestors of clades I–V is clearly the one with 4 metacentric plus 4 submeta- to subtelocentric chromosomes (Fig. 5). This finding confirms earlier hypotheses by Goepfert (1974), who termed this type the “Bauer karyotype” (Bauer, 1954) and regarded it as less derived within the genus. Whether this type is also ancestral for the tribe *Ranunculeae* is awaiting further studies, but the strikingly different  $x=7$  karyotype of *Ceratocephala*, which differs from that of clade VI in *Ranunculus*, is clearly reconstructed as derived.

Among clades I–IV of *Ranunculus*, the karyotype is remarkably stable, despite a great morphological diversity and several incidences of hybridization and polyploidy within these clades. Further karyotype evolution from clade VI upwards drives towards a greater variability, a reduction of the number of metacentric chromosomes together with an increase of chromosomes with unequal arms. In some cases, this happens consecutively within major clades at some terminal taxa (e.g. within clades V and VI). Hence, a reduced number of metacentric chromosomes and increasing asymmetry can be in general regarded as an evolutionarily derived state (Levin, 2002), which is supported by our phylogeny. Increasing asymmetry could be a consequence of

genome contraction (Levin, 2002), as equal DNA reduction per chromosome on both arms will increase asymmetry. At the present stage of knowledge we cannot test this hypothesis as present C value data for *Ranunculus* (<http://data.kew.org/cvalues/>) are too leaky and heterogeneous from the methodical approaches. However, many other processes can influence chromosome evolution, like translocations, rearrangements, and polyploidization with possible genome downsizing (Hörandl and Greilhuber, 2002). We do not have any indications for an ancestral chromosome doubling in any of the clades, as it was observed, e.g. in major clades of *Potamogeton* (Kaplan et al., 2013) and in *Crocus* (Harpke et al., 2013). In *Ranunculus*, all ancestors of major clades were reconstructed as diploid (Hörandl and Emadzade, 2012). Hence, we assume that chromosomal evolution towards unequal arms was in *Ranunculus* not driven by ancient polyploidy, but rather by rearrangements, translocations, fissions or fusions and losses of chromosomes. A similar case (variable basic chromosome numbers, various levels of ploidy, and different karyotypes) is known from the genus *Crepis* (Asteraceae; Babcock, 1947a, 1947b; Jones and Brown, 1976; Dimitrova and Greilhuber, 2000). Loss of chromosomes and chromosomal rearrangements seem to be strong factors causing hybridization barriers and a source for new species.

While the common ancestor of clades V–IX still had the “Bauer karyotype”, the state of the next node in the tree, representing the ancestor of clades VI–IX, remains equivocal (i.e. two or more states are possible). Three main transitions can be observed in the subsequent subclades: (1) preservation of  $x=8$ , mostly with a reduction to 3 metacentric chromosomes in clade IX; (2) the reduction to a basal number of  $x=7$  in clade VI with three metacentric chromosomes in most of the species, and further increase of asymmetry in the annuals *R. chius* and *R. parviflorus*; (3) preservation of the basic number  $x=8$  with a further reduction of metacentric chromosomes to two in clades VII and VIII. Since the backbone of the VI–IX clade was not well resolved, no conclusions can be drawn whether these transitions could have happened stepwise, like in the dysploidy series in *Phyteuma* (Schneeweiss et al., 2013) or via independent origins, as it was observed in basic chromosome numbers of *Crocus* (Harpke et al., 2013).

#### 4.2. Evolutionary and taxonomic relevance

Karyotype evolution further is congruent with gross morphological features. At the genus level, specific karyotypes characterize the genera *Ceratocephala* and *Ficaria*, which were separated from *Ranunculus* based on morphological characters and their phylogenetic position (Emadzade et al., 2010). In *Ranunculus* s.str. the clades I–V with the “Bauer karyotype” share swollen achenes and have as ancestral type of the nectary scale a pocket-like structure (Hörandl and Emadzade, 2012, Appendix 5). The “Bauer karyotype” confirms this paraphyletic group of clades as the stem group of *Ranunculus* (classified as *R.* subgen. *Auricomus*) from which further evolutionary lines emerged. Clade V has a transitional position between the two subgenera, with some morphological characters, the ancestral karyotype and main ecological trends shared with clades I–IV, but being on the base of clades VI–IX according to molecular data (Hörandl and Emadzade, 2012). Clades VI–IX (*R.* subgen. *Ranunculus*), however, share phylogenetically informative morphological structures (flattened achenes, flap-like nectary scales) which are adaptive features connected to a shift to overall more mesic to dry habitats (Hörandl and Emadzade, 2012). Within this subgenus, the diversity and asymmetry of karyotypes is increasing, and sometimes even useful to discriminate otherwise morphologically similar groups (e.g. sections *Polyanthemos* and *Ranunculus*). Congruence of evolution of basic chromosome numbers with molecular phylogenies and morphology-based classifications was also observed, e.g. in the genus *Melampodium*

(Stuessy et al., 2011), but not so in *Crocus* (Harpke et al., 2013), where dysploidy events happened even within major clades and sections. Possibly, karyotype differentiation of major clades, as in *Ranunculus* and *Melampodium*, might prevent hybridization between members of different clades (see below) and help to fix clade-specific character combinations.

#### 4.3. Hybridization and chromosomal evolution

Hybridization is a natural phenomenon and an important power driving evolutionary processes (Stebbins, 1959; Grant, 1981; Stace, 1986; Arnold, 1997; Rieseberg, 1997; Barton, 2001). Chromosomal rearrangements establish powerful crossing barriers between sympatric taxa, as they disturb regular chromosome pairing and bivalent formation at meiosis, thus drastically reducing hybrid fertility (Levin, 2002). Such meiotic disturbances can occur even on the same ploidy level. In homoploid hybrids, chromosomal rearrangements establish immediate crossing barriers against the parental species (e.g. Rieseberg, 1997; Rieseberg et al., 2003). Hence we hypothesize that different karyotypes efficiently establish crossing barriers in *Ranunculus* in crosses involving species with the same ploidy level. Indeed, within the genus *Ranunculus* no hybrids are known between taxa from different clades although experimental crossings have been carried out: Landolt (1954) did not get any hybrid plant crossing species from sect. *Euromontani* (clade IX) with species from the clades IV, VI and VIII; crossing experiments between taxa of clade VI and clade VIII were not successful (Hess, 1955; Baltisberger, 1981); experimental pollinations of species from sect. *Batrachium* (cluster III) with pollen from species of the clades V and VIII revealed no offspring (Cook, 1966). We assume that different chromosomal structures efficiently prevent homoploid hybridization. Members of different clades with different karyotypes can grow together in the same place without any hybridization. For instance, *R. acris* from clade VI (diploid with  $2n=14$ ) frequently occurs in meadows in Central Europe together with members of the *R. auricomus* complex (Clade IV, most meadow species tetraploid with  $2n=32$  and pollen-fertile), but hybrids of these species have never been observed despite overlapping flowering times. *Ranunculus acris* further grows together with *R. bulbosus* and also with *R. nemorosus* (both clade VIII, diploid with  $2n=16$ ) without producing hybrids. In higher altitudes, *R. acris* (clade VI) often co-occurs with species from clade IX in the same subalpine pastures (e.g. *R. montanus*, tetraploid with  $2n=32$ ), but no hybrid has ever been reported. From other, mostly diploid, members of the *R. montanus* group (e.g. *R. villarsii*, diploid with  $2n=16$ ), the different ploidy levels may also act as crossing barrier. *Ranunculus flammula* (clade V,  $2n=32=4x$ ) and *R. repens* (clade VIII,  $2n=32=4x$ ) often co-occur in wet places, but no hybrids have ever been observed. Homoploid hybrids between species from different clades (I–V) sharing the “Bauer karyotype” have not yet been observed in nature as they are usually ecologically and geographically strongly separated from each other. It would be interesting to test hypotheses whether species with the “Bauer karyotype” from different clades could be crossed experimentally.

In contrast, hybridizations within clades occur in natural habitats or could be performed in experiments, supporting a hypothesis that a similar karyotype facilitates hybridization. Based on data from natural habitats as well on experimental crossings, many cases, presented below, are known:

Based on the phylogenetic tree, four sections within clade II can be recognized (Hörandl and Emadzade, 2012). Several homoploid hybrids between taxa of this clade (from natural habitats as well as by experiments) are known though the combinations are not all successful. Several hybrid combinations within and between the two sections *Aconitifolii* and *Ranuncella* are known

(Rouy, 1893; Gautier, 1897; Favarger and Küpfer, 1968; Seitz, 1972; Küpfer, 1974; Huber, 1984, 1985, 1988; Vuille, 1987; Cires et al., 2012, 2014). Successful experimental hybridizations have been performed within and between the other two sections *Epirotes* and *Leucoranunculus* (Müller and Baltisberger, 1984; Baltisberger, 1994). But there are also combinations (some even between taxa of the same section) which did not succeed in experimental crossings: *R. aconitifolius* s.l.  $\times$  *R. seguieri*, *R. aconitifolius*  $\times$  *R. alpestris*, *R. aconitifolius*  $\times$  *R. glacialis* (Huber, 1988), *R. alpestris*  $\times$  *R. seguieri* (Baltisberger and Müller, 1981).

In the phylogenetic tree, *R. cacuminis*, a narrow endemic species known only from the locus classicus on mount Kajmakalan from the border region between Greece and Macedonia, is placed in sect. *Aconitifolii*. *Ranunculus cacuminis* looks very similar to and combines characters of *R. alpestris* (e.g. shape and lobes of leaves) and *R. crenatus* (e.g. venation of leaves, nectary scale). Crossing experiments have been carried out between *R. cacuminis* (sect. *Aconitifolii*) and *R. alpestris* (sect. *Epirotes*) as well as *R. crenatus* (sect. *Leucoranunculus*) (Baltisberger, 1994). In crossings with *R. alpestris* a few achenes were well developed but seeds did not germinate. Crossings with *R. crenatus* yielded more than 150 well developed achenes, the seeds of 14 of which germinated and grew to hybrid plants. This is the only known case of successful (experimental) hybridization between a species from sect. *Aconitifolii* with a species of sect. *Leucoranunculus*. It remains an open question if *R. cacuminis* represents a special case in this aspect or probably belongs to section *Leucoranunculus* rather than to section *Aconitifolii*.

Cluster (clade) III includes three sections (*Batrachium*, *Hecatonina*, *Pseudadonis*). Hybrids between taxa of different sections are not known, and crossing experiments of species of sect. *Batrachium* with *R. sceleratus* (sect. *Hecatonina*) were not successful (Cook, 1966). Section *Pseudadonis* comprises terrestrial alpine species in New Zealand and consequently is ecologically and geographically strongly isolated from the other two sections. But, within section *Pseudadonis*, hybrids seem to be rather frequent (Fisher, 1965; Joly et al., 2009). Hybrids of species of sect. *Batrachium* are in some combinations interfertile (Cook, 1966) and also occur in natural habitats (Roweck, 1981; G. Dahlgren and B. Jonsell in Jonsell and Karlsson, 2001). Within sect. *Hecatonina*, hybrids have been reported between *R. reptabundus* and *R. sceleratus* (P. Uotila in Jonsell and Karlsson, 2001).

Within clade IV, several hybrid combinations are reported. The *R. auricomus* complex is itself a huge hybrid complex (Hörandl et al., 2009), and experimental crosses have been produced between the sexual species, *R. carpaticola*, *R. cassubicifolius* and *R. notabilis*, resulting in hybrids of low fertility or with signs of apomixis (Hörandl, 2008; Hojsgaard et al., 2014). Among arctic species, natural hybrids between *R. pygmaeus* and *R. sulphureus*, *R. nivalis*  $\times$  *R. pygmaeus* and probably *R. nivalis*  $\times$  *R. sulphureus* have been reported from Northern Europe (Roweck, 1981; Ö. Nilsson in Jonsell and Karlsson, 2001). The natural hybrid between *Ranunculus abortivus* and *R. micranthus* (two species from North America) was communicated by Blackwell (1977). In the Himalayan species, frequent interspecific hybridization is inferred from molecular data (Emadzade et al., 2015).

In clade V the hybrid *Ranunculus flammula*  $\times$  *R. reptans* is communicated from natural habitats as well as from experimental crossings (Gornall, 1987; P. Uotila in Jonsell and Karlsson, 2001).

In clade VI the natural hybrid between *R. acris* and *R. uncinatus* has been reported from North America (Benson, 1948). Hess (1953) found hybrids between two other taxa of this clade in a natural habitat in Switzerland and successfully crossed the two taxa experimentally.

Among species of clade VIII, experimental homoploid crossings were fully successful between taxa of the *R. polyanthemus*-group

(Hess, 1955; Baltisberger, 1980). Crossing experiments between taxa of the *R. polyanthemus*-group (2x) with 2x *R. bulbosus* or 2x *R. neapolitanus* (two other members of clade VIII) failed, but crossing experiments between taxa of the *R. polyanthemus*-group (2x) with *R. repens* (4x; member of another subclade within clade VIII) yielded a few hybrids (Baltisberger, 1981). Several species within the subclade comprising the *Ranunculus cantoniensis*-group are involved in hybridization processes (Liao et al., 2008). Hybridization in natural habitats also occurs within the Australian *Ranunculus lappaceus*-group (Briggs, 1962).

In clade IX, hybrids within the sect. *Euromontani* occur in natural habitats and could be produced experimentally (Landolt, 1954, 1956). Additionally experimental hybridization was successful between taxa of sect. *Euromontani* with *R. breyninus* (sect. *Oreophili*, also clade IX) (Landolt, 1954, 1956), but in natural habitats in the Alps, these species do not hybridize even if they grow together. The members of sect. *Ranunculastrum* are separated ecologically and geographically from the two other sections (Hörandl and Emadzade, 2012). Little is known about hybridization within this Mediterranean-Irano Turanian group.

To summarize, karyotype evolution not only reflects the phylogeny of the genus, it may have been a driver of evolutionary processes by rapidly establishing crossing barriers between ancestors of the present major clades. Within these karyotype lineages, hybridization was possible and probably contributed to diversification via allopolyploid and homoploid speciation. Between karyotype lineages, species remained reproductively isolated even in sympatry because of postzygotic crossing barriers.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ppees.2015.11.001>.

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