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# DISSERTATION

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Molecular phylogeny, evolution and biogeography of  
*Ranunculus* (Ranunculaceae) and related genera

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## Co-authorship statement

Chapters 2, 3, 4, and 5 were prepared as papers in international journals. For all of the studies I developed the idea and the methodology. I collected ca. 60 new samples in Iran and the Himalayas, prepared all of the molecular data, performed the analyses and wrote the manuscripts. **Dr. Elvira Hörandl** contributed significantly to project design, interpretation of the results and editing of the manuscripts.

Chapter 2 (Paper 1): was accepted as a manuscript in *Taxon* co-authored with **Dr. Elvira Hörandl**, **Prof. Peter Lockhart** (Massey University, New Zealand) and **Dr. Carlos Lehnebach** (Museum of New Zealand, New Zealand). Dr. Lockhart contributed to the experimental design and helpful discussion particularly in Neighbor Net analysis. Dr. Lehnebach provided some materials, valuable suggestions and he did the histological part.

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## Abstract

The thesis aims at a reconstruction of the phylogeny and biogeographical history of Ranunculeae, with a focus on *Ranunculus* s.str.

*Ranunculus* s.str. is a cosmopolitan genus with approximately 600 species and the largest genus in Ranunculaceae. *Ranunculus* is distributed on all continents and it has a worldwide distribution from the Tropics to the arctic and subantarctic zones. A molecular phylogenetic analysis based on nuclear and plastid markers (ITS, *matK/trnK*, *psbJ-petA*) provides the framework for understanding relationships, biogeography and character evolution in *Ranunculus* and related genera.

Combined molecular data of c. 240 species from all continents reveal a large core clade comprising *Ranunculus* s.str., excluding the small genera *Laccopetalum*, *Krapfia*, *Ceratocephala*, *Myosurus*, *Ficaria*, *Coptidium*, *Beckwithia*, *Cyrtorhyncha*, *Halerpestes*, *Peltocalathos*, *Callianthemoides*, and *Arcteranthis*, but including the water-buttercups and the monotypic genus *Aphanostemma*. Molecular and morphological data reveal that a classification accepting several small genera and one big genus *Ranunculus* s.str. reflects best the phylogeny and the morphological diversity of the tribe. Age estimates based on molecular dating suggest that Ranunculeae diversified between the late Eocene and the late Miocene. Biogeographical analysis suggests a northern hemispheric origin of the tribe and multiple colonization of the S. hemisphere.

Results of biogeographical analyses of *Ranunculus* s.str. support multiple colonizations of all continents. Dispersals between continents must have occurred via migration over land bridges, or via long distance dispersal. In southern Eurasia, isolation of the western Mediterranean and the Caucasus region during the Messinian was followed by range expansions and speciation in both areas. In the Pliocene and Pleistocene, radiations happened independently in the summer-dry W. Mediterranean-Makaronesian and in the E. Mediterranean-Irano-Turanian regions, with three independent shifts to alpine humid climates in the Alps and in the Himalayas.

In previously glaciated areas, rapid colonization was followed by speciation, and regional radiations. This pattern is seen in a clade comprising arctic, Central Asian, North American and European lowland taxa. In North America, the availability of a large area and a broad range of habitats triggered allopatric speciation and adaptive radiation. In contrast, in the Himalayas, the alpine species are restricted to a narrow ecological zone in high altitudes,

resulting in extensive hybridization. The Arctic was colonized multiple times without a pronounced radiation.

Altogether, the success of *Ranunculus* can be referred to a high ability not only to long-distance dispersal to new areas but also to rapid speciation.

## Zusammenfassung

Diese Dissertation hat zum Ziel, eine Rekonstruktion der Phylogenie und der Biogeographie der Ranunculeae mit dem Schwerpunkt auf der Gattung *Ranunculus* s. str. zu erstellen.

*Ranunculus* s.str ist eine kosmopolitische, von tropischen bis in arktische Gebiete verbreitete Gattung und mit ca. 600 Arten die größte innerhalb der Ranunculaceae. Die in dieser Arbeit durchgeführte molekular-phylogenetische Untersuchung von *Ranunculus*, basierend Sequenzen der Kern- und Chloroplasten-DNS (ITS, *matK/trnK*, *psbJ-petA*), bietet die Basis für das Verständnis von Verwandtschaftsverhältnissen, Biogeographie und Evolution innerhalb der Gattung genauso wie zu verwandten Gattungen. Die Kombination dieser molekularen Daten von ca. 240 Arten, gesammelt über das gesamte Verbreitungsgebiet der Tribus, resultierte in einer großen Klade, die *Ranunculus* s.str. beinhaltet, jedoch *Laccopetalum*, *Krapfia*, *Ceratocephala*, *Myosurus*, *Ficaria*, *Coptidium*, *Beckwithia*, *Cyrtorhyncha*, *Halerpestes*, *Peltocalathos*, *Callianthemoides* und *Arcteranthis* exkludiert. Morphologische Untersuchungen unterstützen die Schlussfolgerung, dass eine Gliederung in mehrere kleine Gattungen und eine große Gattung *Ranunculus* s.str. die phylogenetischen Verhältnisse sowie die morphologische Diversität innerhalb der Tribus bestmöglich reflektiert. Die Altersbestimmung der Ranunculeae basierend auf molekular-genetischen Datierungsmethoden deutet darauf hin, dass die Diversifizierung der Tribus höchstwahrscheinlich zwischen dem späten Eozän und dem späten Miozän erfolgt ist. Biogeographische Analysen weisen auf einen nordhemisphärischen Ursprung der Tribus und darauf folgende mehrfache Kolonialisierung der Südhemisphäre hin. Die Verbreitung zwischen den Kontinenten fand sowohl durch Fernverbreitung als auch durch Vikarianz statt. In Vorderasien folgten der Isolation des westlichen Mediterrangebietes vom Kaukasusgebiet während des messinischen Zeitalters mehrere Zyklen von Arealerweiterungen und Artbildung in beiden Gebieten. Während des Pliozäns und des Pleistozäns fanden Radiationen innerhalb der Tribus unabhängig voneinander in westmediterran-makaronesischen Gebieten und in der sommertrockenen, ostmediterranen Irano-Turanischen Region statt. Dabei erfolgten drei voneinander unabhängige ökologische Wechsel zu humiden Klimaten in den Alpen und im Himalaya-Gebirge. In eiszeitlich vergletscherten Gebieten kam es nach der letzten Eiszeit zu sehr schnellen Kolonisierungen, und damit zur vermehrten Artbildung und zu lokalen Radiationen. Dieses Muster spiegelt sich in einer Klade, die sowohl arktische, zentralasiatische, nordamerikanische Gebirgssippen als auch europäische Tieflandsarten

beinhaltet. In Nordamerika führte die Verfügbarkeit verschiedenster Habitate innerhalb eines sehr großflächigen Areals zur allopatrischen Speziation und adaptiver Radiation. Im Gegensatz dazu sind die Arten im Himalayagebiet ausschließlich in einer sehr engen ökologischen Zone im hochalpinen Bereich zu finden, wodurch verstärkt Hybridisierung erfolgt. Die Arktis wurde mehrfach und ohne deutliche Radiationen kolonisiert.

Zusammenfassend lässt sich aus dieser Arbeit schließen, dass die Gattung *Ranunculus* nicht nur das Potential zur globalen Fernverbreitung, sondern auch zur raschen Artbildung aufweist.

# Chapter 1

## General Introduction

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### i. Ranunculeae

Ranunculeae DC. (Ranunculoideae Hutch.) has been classified as one of ten tribes of Ranunculaceae based on molecular and morphological data by Wang *et al.* (2009). The tribe includes sixteen genera and it is distributed in all continents (Tamura, 1995). Ranunculeae have a cosmopolitan, mainly extratropical distribution. *Ranunculus* is the only genus distributed in all continents. Most of the other genera in the tribe have very restricted distributions and many of the monotypic genera are endemic to one continent such as *Cyrtorhyncha* and *Kumlienia* (North America), *Laccopetalum* (South America), and *Peltocalathos* (South Africa). Most species of this tribe are adapted to temperate and cold climates and are found in mountain regions of the world.

There are only a few worldwide studies on Ranunculeae (e.g. Candolle, 1824; Prantl, 1887; Tamura, 1993 and 1995) and different classifications for its members have been proposed. Candolle (1817) described Ranunculeae based on floral features, underground parts, and achenes. The most up to date and worldwide classification of the tribe is that by Tamura (1995) based on differences in the structure of achenes. Previous classifications often included the genera *Ficaria*, *Coptidium*, and *Arcteranthis* in *Ranunculus* (Candolle, 1824; Prantl, 1887) or excluded *Batrachium* from *Ranunculus* (Janchen, 1958; Rostrup, 1958; Wang and Gilbert, 2001). Tamura (1995) segregated several small genera from *Ranunculus*. He subdivided Ranunculeae into three subtribes: Trautvetteriinae, Myosurinae and Ranunculinae included *Trautvetteria*, *Myosurus* and 14 genera, respectively (Tamura, 1995). Tamura excluded several species from *Ranunculus* such as *Aphanostemma*, *Callianthemoides*, and *Krapfia* and classified them as separate genera.

A number of molecular phylogenetic studies within the Ranunculaceae suggested that Ranunculeae are monophyletic (Hoot, 1995; Hoot *et al.*, 2008; Johansson, 1995, 1998; Ro *et al.*, 1997; Hörandl *et al.*, 2005; Lehnebach *et al.*, 2007; Wang *et al.*, 2009). Previous phylogenetic studies of this tribe based on incomplete sampling did not reveal complete congruence with Tamura's classification (Hörandl *et al.*, 2005; Paun *et al.*, 2005). Hörandl *et al.* (2005) suggested that this incongruence could be due to morphological adaptations of species to different habitats. Previous phylogenetic studies using nuclear ribosomal DNA

sequences (Hörandl *et al.*, 2005) and chloroplast DNA sequences (Paun *et al.*, 2005) showed that some sections of *Ranunculus* were not nested in the *Ranunculus* core clade like the sections *Coptidium* and *Ficaria*.

The origin of Ranunculaceae probably dates back to the mid Eocene (Paun *et al.*, 2005; Hoffmann *et al.*, 2010). However, previous age estimates for the tribe suffered from incomplete sampling of genera and the lack of internal calibration points. Therefore, the timing of biogeographical events has remained tentative. Up to now a complete taxon sampling, a comprehensive framework for the classification, a reliable molecular dating, and a worldwide biogeographical study of this tribe based on molecular and morphological data, was missing.

## ii. *Ranunculus*

*Ranunculus* L. with over 600 species is the largest genus of the Ranunculaceae (Tamura, 1993; 1995). *Ranunculus* is distributed on all continents and it has a worldwide distribution from the Tropics to the arctic and subantarctic zones. The genus is especially species-rich in temperate to meridional zones (e.g., Ovczinnikov, 1937; Iranshahr *et al.*, 1992; Whittemore, 1997). In the tropical areas, species are restricted to high mountain areas (e.g., African species; Tamura, 1993, 1995; Gehrke and Linder, 2009). Species of *Ranunculus* are established in a variety of wet to dry habitats from the lowland to high alpine zones and show several morphological adaptations to different habitats (Paun *et al.*, 2005; Emadzade *et al.*, submitted). *Ranunculus* shows different levels of polyploidy, which is sometimes connected to apomixis (Hörandl *et al.*, 2005). Two basic chromosome numbers were identified in buttercups,  $x = 8$  and  $x = 7$ , whereby the latter is rare. Hybridization was recorded in many groups, such as in the *Ranunculus polyanthemus* group (Baltisberger, 1980), *Ranunculus* subg. *Batrachium* (Cook, 1963; Hörandl *et al.*, 2005), and the alpine species of New Zealand (Lockhart *et al.*, 2001). Lockhart *et al.* (2001) and Hörandl *et al.* (2005) suggested that hybridization and polyploidy could be important factors for the diversification and evolutionary success of *Ranunculus*.

Monophyly of *Ranunculus* has been assumed by previous molecular phylogenetic studies (Hoot, 1995; Johansson, 1995, 1998; Ro *et al.*, 1997; Hörandl *et al.*, 2005; Paun *et al.*, 2005; Lehnebach *et al.*, 2007; Gehrke and Linder, 2009; Hoffmann *et al.*, 2010). Previous studies (using cpDNA restriction sites, Johansson, 1998; ITS sequences, Hörandl *et al.*, 2005; matK/trnK plus ITS, Paun *et al.*, 2005; Lehnebach, 2008; Gehrke and Linder 2009; Hoffmann *et al.*, 2010) showed that the core *Ranunculus* clade was subdivided into several well-

supported clades that corresponded to widespread ecological groups (e.g., wetland and aquatic species) or to regional geographical groups (e.g., in the European mountain system; Hörandl *et al.*, 2005; Paun *et al.*, 2005). However, all previous studies included only an incomplete sampling.

Here it has been tried to reconstruct the phylogeny of *Ranunculus* and allied genera with a worldwide sampling from all continents (except Australia). In particular, Eurasian species have been collected intensively to understand the diversification in this area in great details. The combination of phylogenetic data with spatial-temporal data provides a strong hypothesis for understanding the biogeographical history of the group (Hunn and Upchurch, 2001; Donoghue and Moore, 2003; Kelly *et al.*, 2009). Therefore I combine here the results from molecular dating and biogeographical analyses to provide a comprehensive hypothesis of the biogeographical history of *Ranunculus*.

### **iii. Biogeography**

Biogeography is the study of the distributions of organisms in space and time. This scientific discipline tries to find out spatial patterns of biological diversity (Lomolino *et al.*, 2006) and to answer questions such as: Why is a taxon limited to its present area? How have historical events shaped the distribution of a taxon?

During the past 30 years, this research field has undergone many changes. For instance, before 1960, most biogeographers believed that ancestors of species dispersed across barriers, then became isolated, and evolved into new species (Udvardy, 1969). For centuries, dispersal was the dominant explanation for the distribution of organisms, but the emergence of plate tectonics, the spread of cladistic thinking, and the development of phylogenetic systematics made vicariance an important biogeographical hypothesis (Wiley, 1998; de Queiroz, 2005). After the 1970s, the vicariance school proposed that the main way in which biodiversity was generated was through the fragmentation of widespread ancestors by the emergence of a geographical barrier (Croizat *et al.*, 1974; Nelson and Platnick, 1981; Craw *et al.*, 1999; Humphries and Parenti, 1999). Despite many counter arguments, vicariance became the dominant hypothesis of historical biogeography (Morrone and Crisci, 1995).

Theoretically, dispersalists believed that the common ancestor originally occurred in one of the areas and dispersed later to other areas or in other words, the centre of origin corresponds to the centre of diversity. In contrast, the vicariance interpretation assumed that the ancestor was originally widespread and later split by a geographical barrier. Its descendants have survived till present, or in other words, species would originate only by allopatric speciation.

Therefore, the centre of diversity is not the centre of origin (Nelson and Platnik, 1984). In fact, disjunct distributions can be explained either by fragmentation of widespread ancestors by vicariant (isolating) events or by dispersal across a barrier. However, recent paleogeographical evidence showed that many areas have a more complicated geological history than a simple event of separating landmasses (Ronquist, 1997; Sanmartin and Ronquist, 2004).

Molecular-based phylogenetic studies and estimates of the divergence times of lineages supported the role of dispersal as a primary process shaping distribution patterns (Voelker, 1999, 2002; de Queiroz, 2005). These data provide a huge amount of evidence supporting a hypothesis of transoceanic dispersal versus vicariance (Givnish and Renner, 2004; Sanmartin and Ronquist, 2004; de Queiroz, 2005).

To understand the biogeographical history of any group, information from both spatial and temporal dimensions is necessary (Hunn and Upchurch, 2001; Donoghue and Moore, 2003; Kelly *et al.*, 2009). Precise temporal calibration of phylogenies allows researchers to test the effect of past climatic and geological changes on the evolutionary dynamics and distribution patterns of organisms. If the dating of the divergence between the disjunctly distributed lineages to a time is older than the emergence of a geographical barrier one can argue for vicariance; in contrast, if dating of this divergence is younger than the emergence of a geographical barrier, this event can be explained by long-distance dispersal (Kropf *et al.*, 2006). The number of suggested long-distance dispersal events in the biogeographical history of flowering plants has been recently increased (de Queiroz, 2005). Due to passive dispersal in plants the occurrence of long distance dispersal or transoceanic dispersal in plants is not unlikely. Smith (1986) showed that only one successful long-distance dispersal and establishment needs to occur approximately every 10,000 years to explain the species richness observed in the Australasian alpine and tropic-alpine flora. On the other hand, Berg (1983) pointed out that long distance dispersal does neither need to be frequent nor regular to be effective.

#### **iv. Diversification and speciation**

Geographical isolation is not the only mode of speciation in *Ranunculus*. Besides allopatric, parapatric, and sympatric speciation, hybrid (homoploid, polyploidy), ecological, and asexual speciation has to be considered (Futuyma, 2005). However, in the real life, more than one model often needed to accommodate diversity and so many types of taxa that have originated through many different types of processes (Stuessy, 2008). Geographical speciation long

regarded as the most common mode of speciation. Definition of different kind of geographical speciation is related to level of gene flow between taxa: totally absent gene flow, allopatric speciation; no physical barriers to gene flow, sympatric speciation; and intermediate scenarios, parapatric speciation (Nosil, 2008). There is growing evidence that ecological selection on traits such as environmental tolerance or reproductive timing, has an important role in the divergence and speciation (Levin, 2005), although, certainly it could act with other models.

Another important mode of speciation especially in plants is hybrid speciation. Hybrids can share habitats with their parents (sympatric speciation) or occupy new areas that are extremely different from the parental species (allopatric and parapatric speciation; e.g. Rieseberg and Willis, 2007). For a long time it has been accepted that because of a great genetic variability and a greater ecological adaptability (reviewed by Brochmann *et al.*, 2004; Rieseberg and Willis, 2007; Mallet, 2007), polyploids are better adapted to extreme habitats than diploids (Hagerup, 1932). Hybridization and polyploidization create without any doubt “genomic novelty” (e.g., Comai, 2005; Chen, 2007). In this respect, hybridization and polyploidization can be seen as modes of rapid speciation (Grant, 1981; Soltis *et al.*, 2004).

Previous studies showed hybridization occurs within many sections of *Ranunculus* s.str. such as water-buttercups (*R.* subg. *Batrachium*; Cook, 1963), the alpine species of New Zealand (*R.* sect. *Pseudadonis*; Fisher, 1965; Lockhart *et al.*, 2001). Hörandl *et al.* (2005) based on sequences of the nrITS revealed complex patterns of relationship and suggested hybridization in the apomictic *R. auricomus* complex, arctic-high alpine species of the North America and Eurasia, and *R.* subg. *Batrachium*. Molecular dating approaches suggested diversification of most of these sections and clades already in the Pliocene-Pleistocene (Paun *et al.* 2005), which probably was affected by Quaternary climatic fluctuations. Much of the current distribution range of *Ranunculus* in arctic and alpine areas includes previously glaciated regions in the North American Mountains, the Central Asian Mountains, the Himalayas, and the Arctic.

## **v. Aims and outlines of this thesis**

A broad phylogenetic analysis of more than one third species of Ranunculaceae and *Ranunculus* from all continents is presented in this study using DNA sequences of the internal transcribed spacer region (ITS) and chloroplast markers (*matK/trnK* and *psbJ-petA*). We combine here the results from molecular phylogeny, molecular dating, and biogeographical analyses.

The main aims of this study are (i) to investigate the phylogenetic relationships within *Ranunculus* and allied genera using molecular data, (ii) to present a revised classification for Ranunculeae based on molecular and morphological data, (iii) to reconstruct divergence dates within Ranunculeae and *Ranunculus*, (iv) to develop hypotheses for the spatial distribution of buttercups and allied genera, (v) to point out the main migration routes, (vi) to reconstruct the main factor(s) shaping the modern distribution of the *Ranunculus* and tribe, and (vii) to develop a phylogenetic framework for elucidating the processes of spatial and temporal diversification of arctic and alpine *Ranunculus* in the Northern Hemisphere.

This dissertation is arranged in the order of four manuscripts (one in press, three submitted and in review). The focus of first paper is to investigate the phylogenetic relationships within Ranunculeae using molecular data. This study identifies morphological synapomorphies and diagnostic characters useful for the classification of genera and provides a revised classification of the tribe. The second paper is concentrated on the reconstruction of divergence dates within the tribe. This paper localizes the center of origin for the tribe and the main migration routes including the relative role of long-distance dispersal and vicariance. The topic of the third paper is to provide a hypothesis of the biogeographical history of *Ranunculus* in the meridional to temperate zones. I investigate the main migration routes between continents and areas of diversity to reconstruct the main factor(s) shaping the modern distribution of the genus. In the fourth paper I tried to reconstruct the evolutionary history of buttercups in previously glaciated areas. By comparison of the North American Mountain chains and the Himalayas, I analyzed whether rapid speciation in these areas was caused by adaptive radiation and ecological crossing barriers, or by hybridization and polyploidy.

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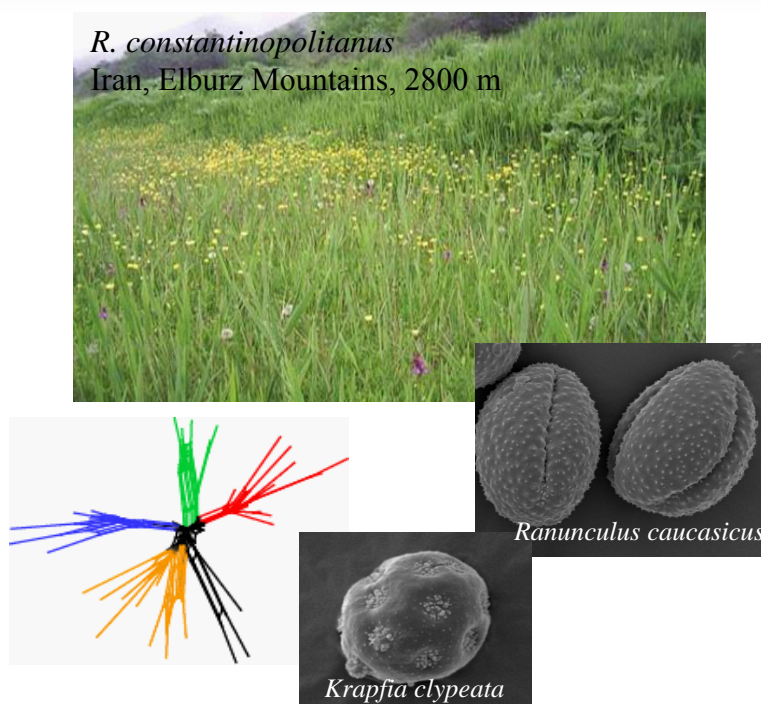
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## Chapter 2

### A molecular phylogeny, morphology and classification of genera of Ranunculeae (Ranunculaceae)\*

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## Abstract

Ranunculeae represent a highly diverse and cosmopolitan tribe within Ranunculaceae. Because of the great diversity of morphological features and lack of molecular phylogeny for the tribe, the classification of its genera has always been controversial. We report here molecular phylogenetic analyses based on nuclear and plastid markers (the ITS of the nuclear ribosomal DNA, the *matK* gene, the flanking *trnK* region and the highly variable *psbJ-petA* region) that provide a framework for understanding relationships and character evolution within the tribe. Maximum parsimony analyses of these molecular data suggest a weakly supported basal dichotomy within the tribe, while Neighbor Net analysis indicates strong support for five distinct lineages. Both methods of analysis revealed several well-supported small terminal clades which correspond to previously described genera, which are characterised by unique morphological features and character combinations. Anatomical structures of the achenes (sclerenchyma layer, venation pattern, microstructure of the surface) suggested relationships with greatest concordance to those in the molecular phylogeny. Macroscopic analysis of achene morphology often indicated parallel evolution of structures related to certain dispersal mechanisms. Characters of the perianth, the androecium, the gynoecium and the pollen are overall highly homoplasious, but several distinct features characteristic of small terminal clades and terminal branches can be observed. Geographic isolation and adaptations to certain habitats may have triggered the evolution of specific morphological features. We conclude that a classification accepting several small genera (*Arcteranthis*, *Beckwithia*, *Callianthemoides*, *Ceratocephala*, *Coptidium*, *Cyrtorhyncha*, *Ficaria*, *Halerpestes*, *Hamadryas*, *Krapfia*, *Kumlienia*, *Laccopetalum*, *Myosurus*, *Oxygraphis*, *Paroxygraphis*, *Peltocalathos*, and *Trautvetteria*) and a big genus *Ranunculus* s.str. (including the former genera *Batrachium*, *Aphanostemma* and *Gampsoceras*) reflects best the molecular phylogeny and the morphological diversity of the tribe.

**Keywords:** anatomy, Ranunculeae, molecular systematics, morphology, SEM, taxonomy.

## INTRODUCTION

Ranunculaceae are a large plant family with a worldwide distribution. This family has been considered as one of the most basal families within the eudicots (Soltis & al., 2005; Simpson, 2006; Heywood & al., 2007) and its crown age has been estimated as c. 75 my (Anderson & al., 2005). The family shows a wide variation in morphological characters, especially in fruit types, and in its floral organization. Several classifications have been proposed for Ranunculaceae based on morphological characters (Hutchinson, 1923; Janchen, 1949; Tamura, 1995), on molecular data (Jensen & al., 1995; Ro & al., 1997), and on a combined molecular and morphological dataset (Wang & al., 2009). From the conventional characters used, chromosome type and base number have been found to be most congruent with the phylogeny of the family as inferred from molecular data (Ro & al., 1997; Wang & al., 2009). Recent molecular studies have given insights into the phylogenetic relationships within this family (Johansson & Jansen, 1993; Johansson, 1995; Hoot, 1995; Kosuge & al., 1995; Jensen & al., 1995; Ro & al., 1997; Ro & al., 1999; Wang & al., 2005; 2009).

The family has been subdivided into three subfamilies and eleven tribes by Tamura (1995). This classification has been based on chromosome base number, carpel and fruit types. The tribe Ranunculeae DC., in the subfamily Ranunculoideae Hutch., includes about 650 species and it is distributed in all continents (Tamura, 1995). A number of molecular phylogenetic studies within the Ranunculaceae suggest that this tribe is monophyletic (Hoot, 1995, Hoot & al., 2008; Johansson, 1995, 1998; Ro & al., 1997; Lehnebach & al., 2007; Wang & al., 2009). The tribe has unitegmic ovules as in Anemoneae and Callianthemaeae (sensu Wang & al., 2009), but in Ranunculeae ovules are ascending (except *Myosurus* which has pendent ovules; Tamura, 1995). Petals in Ranunculeae have at least one nectary gland near the base. There are only a few worldwide studies on Ranunculeae (e.g. Candolle, 1824; Prantl, 1887; Tamura, 1993 & 1995) and different classifications for its members have been proposed (Table 1). Discrepancies between these classifications are probably due to the ample variation in floral characters, e.g. bisexual or unisexual flowers, petaloid or sepaloid sepals and the presence or absence of petals. Candolle (1817) described Ranunculeae based on floral features, underground parts, and achenes. In his classification, Ranunculeae comprised four genera: *Myosurus*, *Ranunculus*, *Ceratocephala* and *Ficaria* (Table 1).

Prantl (1887) based the classification of genera on features of fruits and the perianth, and treated *Myosurus*, *Ranunculus*, *Trautvetteria*, and *Oxygraphis* as closely related genera (Table 1). Although his study had a worldwide coverage, several South American taxa were not

included. The most up to date and worldwide classification of the tribe is that by Tamura (1995) based on differences in the structure of achenes. In his classification, 16 genera were included in the tribe (Table 1) and these were grouped into three subtribes; Trautvetteriinae without petals (one genus), Myosurinae with a spur-like projection at the base of the sepals and pendulous ovules (one genus) and Ranunculinae (14 genera).

Delimitation of *Ranunculus* L., the largest genus in Ranunculinae and closely related genera has been a source of debate for centuries. Previous classifications often included the genera *Ficaria*, *Coptidium*, and *Arcteranthus* in *Ranunculus* (Candolle, 1824; Prantl, 1887) or excluded *Batrachium* from *Ranunculus* (Janchen, 1958; Rostrup, 1958; Löve & Löve, 1961; Wang, 2001). Tamura (1995) segregated several small genera from *Ranunculus* (Table 1) and used characters from reproductive structures, such as achene and petals to establish generic boundaries. Although a number of studies have assessed the diversity of achenes (Trzaski, 1999), petals (Parkin, 1928), nectary scales (Benson, 1940), karyotypes (Goepfert, 1974), and pollen structure (Santisuk, 1979) within Ranunculeae, none have revealed individual characters diagnostic for delimitation of the genera. In combination some characters are potentially informative for identifying genera (Tamura, 1995), however little insight has been gained from morphological analyses regarding relationships among genera.

A number of molecular investigations of the Ranunculeae and its members are currently available (e.g. Johansson, 1998; Hörandl & al., 2005; Paun & al., 2005; Lehnebach & al., 2007; Hoot & al., 2008; Gehrke & Linder, 2009; Hoffmann & al., 2010). These studies included c. 200 species covering all sections and subgenera of *Ranunculus* sensu Tamura (1995), with the exception of *R. pinardii* (*R.* subg. *Gampsoceras*), and *R.* sect. *Ficariifolius* L. Liou., and have provided a comprehensive phylogenetic framework for the species of *Ranunculus* s.str. These phylogenetic studies have revealed that the water-buttercups, *Batrachium* (= *R.* sect. *Batrachium*) are nested within *Ranunculus* s.str.; and that *Aphanostemma* (= *R. apiifolius*) is a monotypic genus nested within *Ranunculus* s.str. (Hörandl & al., 2005; Paun & al., 2005; Lehnebach & al., 2007). Unfortunately, none of these studies have included all genera of the tribe (as delimited by Tamura, 1995) and phylogenetic relationships between some of the genera are still unknown. The tree topologies of previous molecular studies on the genus *Ranunculus* and allied genera (Johansson, 1998; Hörandl & al., 2005; Paun & al., 2005; Lehnebach & al., 2007; Hoot & al., 2008; Gehrke & Linder, 2009; Hoffman & al., 2010) have revealed the position of *Ficaria* separate from the *Ranunculus* clade and all (except for Johansson, 1998) have supported the inclusion of *Myosurus* within Ranunculeae. The separation of *Coptidium* from a core *Ranunculus* clade is

evident in Johansson (1998), Hörandl & al. (2005), Paun & al. (2005), Lehnebach & al. (2007), Gehrke & Linder (2009), and Hoffman & al. (2010). Results of Hörandl & al. (2005) placed *Arcteranthis*, *Callianthemoides*, *Halerpestes*, *Oxygraphis*, and *Peltocalathos* on basal branches and supported Tamura's (1995) classification of separate genera. The analyses of Hoot & al. (2008) suggested an exclusion of *Hamadryas* from the core *Ranunculus* clade, while Lehnebach & al. (2007) accepted *Krapfia* and *Laccopetalum* as sister taxa to *Ranunculus* s.str. However, some authors refrained from final taxonomic conclusions at the generic level because of incomplete sampling of taxa or markers (Johansson, 1998; Hörandl & al., 2005; Hoot & al., 2008). For some taxa of the tribe, molecular data were not available (*Kumlienia hystricula*, *Cyrtorhyncha ranunculina*, and *Gampsoceras pinardii*). A comprehensive framework for the classification of this tribe based on morphology and a complete molecular phylogeny has not yet been published.

Ranunculeae have a cosmopolitan, mainly extratropical distribution with *Ranunculus*, being the only genus distributed in all continents. Most of the other genera in the tribe have very restricted distributions and many of the monotypic genera are endemic to one continent, e.g. *Arcteranthis* (northwestern North America), *Cyrtorhyncha* (western North America), *Kumlienia* (western North America), *Krapfia* and *Laccopetalum* (northern Andes in South America) and *Peltocalathos* (southern South Africa). Other genera, such as *Ceratocephala*, *Myosurus*, and *Ficaria* are mainly distributed in the northern hemisphere, but seem to have extended their distribution rather recently (Tamura, 1995). Only *Halerpestes*, occurring in Asia, South and North America, and the cosmopolitan genus *Ranunculus* have larger distribution areas. The different taxonomic treatments of genera in regional floras (Table 1) have hampered so far a worldwide classification of the tribe. Most species of this tribe are adapted to temperate and cold climates and are found in mountainous regions of the world.

The main aims of this study were to (1) to investigate the phylogenetic relationships within Ranunculeae using molecular data, (2) to identify morphological synapomorphies and diagnostic characters useful for the classification of genera, and (3) to provide a revised classification of the tribe. Unlike previous studies, we examined morphological characters including type of pollen aperture, achene surface and shape of the nectar. We studied these features within a phylogenetic framework provided by analyses of the internal transcribed spacer region (ITS) and chloroplast markers (*matK/trnK* and *psbJ-petA*). This comparative approach provided a means to evaluate character evolution terms of its significance for ecology and systematics (Stuessy, 2003).

## MATERIALS AND METHODS

**Plant material.** -- Fifty-two taxa representing 16 of the 17 genera Tamura (1993, 1995) included in the Ranunculeae were sampled (Table 1). Only the genus *Paroxygraphis* was not included because material was not available. Except for monotypic genera, at least two species for each genus were studied. At least two species were studied from each of the clades and subclades identified for *Ranunculus* s.str. in previous studies (Hörandl & al., 2005; Paun & al., 2005; Emadzade, unpubl.). We included also *Ranunculus pinardii*, a species which has been described as a distinct monotypic genus, *Gampsoceras* (Steven, 1852), and one representative of sect. *Ficariifolius* sensu Tamura (*R. cheirophyllus*). *Anemone* and *Isopyrum* were chosen as outgroup taxa. The first one belongs to the tribe *Anemoneae*, sister to the tribe Ranunculeae (Hoot & al., 2008; Wang & al., 2009) and the second one to the tribe *Thalicthroideae* which is distantly related to Ranunculeae (Hoot & al., 2008; Wang & al., 2009). Voucher information and GenBank accession numbers are provided in Appendix 1.

**Morphological characters.** -- Based on herbarium material and literature data, 33 morphological characters were investigated (Ovczinnikov, 1937; Benson, 1940; Davis, 1960; Goepfert, 1974; Riedl & Nasir, 1990; Iranshahr & al., 1992; Rau, 1993; Tutin & Cook, 1993; Whittemore, 1997; Wang & Gilbert, 2001). We have indicated the character states in Table 2, and how they were scored in Appendix 2. Selected characters were mapped, using MacClade v. 4.0 (Maddison & Maddison, 2000), onto a tree topology inferred using concatenated sequence data.

Surface of the achenes and type of apertures of the pollen grains were studied with a scanning electron microscope (SEM). Samples taken from herbarium specimens were glued to aluminium stubs, and coated with gold (BALZERS Sputter Coater). The samples were viewed and photographed on a SEM, JEOL JSM-6390 at 10 kV at the Faculty Center of Biodiversity, University of Vienna. Pollen aperture types were coded following the terminology of Santisuk (1979). For histological observations, achenes were fixed in Alcohol-Formalin-Acetic acid solution overnight and then dehydrated using ethanol series and embedded in Paraplast. Sections of 10 µ thick were obtained with the microtome, stained with Toluidine blue and later mounted. Ranunculeae have a single-seeded, indehiscent dry fruit with a hardened pericarp. Because of numerous definitions and applications of fruit terms in the literature (e.g. achene, utricle, nutlet) their description can be ambiguous. “Achene” here is treated as “*An indehiscent pericarpium, or fruit, with a pericarp contiguous to the seed(s)*” (Spjut, 1994; Simpson, 2006). The space between seed and pericarp in the fruits of

Ranunculeae is variable. However, we use the term achene for the fruit of the Ranunculeae as do most modern authors (Tamura, 1993, 1995; Simpson, 2006). Ontogenetic studies have shown that nectary organs in Ranunculaceae are derived from stamens (e.g., Erbar & al., 1998). In most genera of Ranunculeae, tepals have the function of a calyx and the petals. They are called “honey-leaves” (Prantl, 1887) or “nectar-leaves” (Janchen, 1949), and have an insect-attracting function. In this study we apply the commonly used terms sepals and petals for the two whorls of the perianth (Ovczinnikov, 1937; Tamura, 1995; Whittemore, 1997).

**DNA extraction, amplification, and sequencing.** -- Total genomic DNA from silica-dried or herbarium material was extracted using a modified CTAB technique (Doyle & Doyle, 1987). The whole internal transcribed spacer (ITS, including ITS1, the 5.8 S rDNA, ITS2) was amplified as a single piece with primers ITS 18sF and ITS 26sR (Gruenstaeudl & al., 2009) or in the case of degraded DNA from poor quality herbarium tissue, in two pieces with additional primers (ITS 5.8sF and ITS 5.8sR) as internal primers (Gruenstaeudl & al., 2009). Sequencing of the *matK/trnK* region was performed according to the protocol described by Paun & al. (2005). Amplification of the non-coding *psbJ/petA* region carried out as a single piece in all samples with using *psbJ* and *petA* primers of Shaw & al. (2007). PCR was performed in 23 µl reactions containing 20 µl 1.1× Reddy Mix PCR Master Mix (including 2.5 mM MgCl<sub>2</sub>; ABgene, Epsom, UK), 1 µl each primer (10 mmol/L) and 1 µl template DNA. 1 µl of 0.4% bovine serum albumin (BSA, Promega, Madison, WI, U.S.A.) for *matK* and *psbJ-petA*, and in the case of the ITS region, dimethyl sulfoxide (DMSO) was added to reduce problems associated with DNA secondary structure. PCR products were purified using *E. coli* Exonuclease I and Calf Intestine Alkaline Phosphate (CIAP; MBI-Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions. Cycle sequencing was performed using Big Dye<sup>TM</sup> Terminator v3.1 Ready Reaction Mix (Applied Biosystems), using the following cycling conditions: 38 cycles of 10 sec at 96°C, 25 sec at 50°C, 4 min at 60°C. All DNA regions were sequenced in both directions. The samples were run on a 3130xl Genetic Analyzers capillary sequencer (Applied Biosystems).

**Sequence alignment and phylogenetic analysis.** -- The sequences of all markers were initially aligned using Clustal X (Thompson & al., 1997). Subsequent corrections were carried out manually using BioEdit version 7.0.9.0 (Hall, 1999). Indels were treated as binary characters following the “simple indel coding method” (Simmons & Ochoterena, 2000) using the program SeqState version 1.36 (Müller, 2005). Due to degraded DNA from poor quality herbarium tissue and difficulties in amplification of DNA, we could neither sequence the

*matK/trnK* region in *Arcteranthus* nor the *psbJ-petA* region in *Krapfia* and *Myosurus*. Thus these absent sequences were scored as missing data. The *psbJ-petA* sequence of *Ceratocephala* was extremely highly diverged and could not be aligned to the other species. High relative levels of divergence for *Myosurus* and *Ceratocephala* were also reported in the cpDNA restriction site analyses of Johansson (1998). Since the tree building assumption of similar evolutionary constraint (Lockhart & Steel, 2005) appears violated in the *psbJ-petA* sequences for these taxa we excluded this region for these species from the analysis. Nuclear and chloroplast sequences were analyzed separately and in combination. A heuristic search for the most parsimonious (MP) trees was performed with PAUP\* version 4.0b8 (Swofford, 2002). The analyses involved 1000 replicates with stepwise random taxon addition, tree bisection–reconnection (TBR) and branch swapping saving no more than 10 trees per replicate. All characters were equally weighted and treated as unordered (Fitch, 1971). Internal branch support was estimated using non-parametric bootstrapping (Felsenstein, 1985) with 10,000 bootstrap replicates each with 10 random sequence addition replicates holding maximally 10 trees per replicate, SPR branch swapping, and MulTrees on. Since our phylogenetic reconstructions indicated numerous relationships where bootstrap support was <50%, we were interested to determine whether this was due to conflicting support or absence of phylogenetic signal. Phylogenetic network methods (Huson & Bryant, 2006) provide a means of evaluating the extent to which data exhibits a hierarchical structure. Interestingly, non-hierarchical data structure has been inferred frequently in *Ranunculus* by using split decomposition (Lockhart & al., 2001; Hörandl & al., 2005). However, for larger datasets, the Neighbor Net method often provides better resolution than split decomposition due to the criterion it uses to calculate support for relationships among taxa. Like Split decomposition, Neighbor Net also calculates the support for “splits” (relationships) from distances and displays these splits in a graph (i.e. a “splits graph” or “split network”). While split decomposition uses the criterion of “weak compatibility” (Lockhart & al., 2001) in identifying splits, Neighbor-Net uses an algorithm that determines a circular ordering of taxa (i.e., based on the extent of differences between their sequences the taxa are ordered around a circle). The layout on the circle determines what splits occur in the data and can be displayed in a planar graph. The support for each of these splits is then measured using a least squares method that adjusts the lengths of the splits in the splits graph so as to minimize the difference with the pairwise distances in the original data matrix (Bryant & Moulton, 2004; Huson & Bryant, 2006). Non tree-like splits graphs indicate contradictory support for relationships. Phylogenetic error, hybridisation and horizontal gene transfer can all potentially contribute to

the non tree-like nature of splits graphs (Bryant & Moulton, 2004). We used the Neighbor Net analysis implemented in SplitsTree4 version 4.10 (Huson & Bryant, 2006), applying Hamming distances with gaps and ambiguous sites coded as missing data. Bootstrap support for internal splits (which define clusters) was calculated with 1000 replicates.

Table 1. Summary of classifications of Ranunculeae.

Genera accepted in this study (worldwide)	Whittemore (1997, North America)	Tamura (1995, worldwide)	Prantl (1887, worldwide)	Ovczinnikov (1937, USSR)	De Candolle 1824
<i>Arcteranthis</i> Greene	<i>R. subg. R. sect. Arcteranthis</i>	<i>Arcteranthis</i>	-	-	-
<i>Beckwithia</i> Jeps.	<i>R. subg. Crymodes</i>	<i>R. subg. Crymodes</i>	<i>R. sect. Hypolepium</i>	-	-
<i>Callianthemoides</i> Tamura	-	<i>Callianthemoides</i>	-	-	-
<i>Ceratocephala</i> Moench	<i>R. subg. Ceratocephalus</i>	<i>Ceratocephala</i>	<i>R. sect. Ceratocephalus</i>	<i>Ceratocephala</i>	<i>Ceratocephala</i>
<i>Coptidium</i> Rydp.	<i>R. subg. Coptidium &amp; Pallasiantha</i>	<i>R. subg. Coptidium &amp; Pallasiantha</i>	<i>R. sect. Marsypadenium</i>	<i>R. subg. Auricomus sect. Coptidium</i>	<i>Ranunculus</i>
<i>Cyrtorhyncha</i> Torr. & A. Gray	<i>R. subg. R. sect. Cyrtorhyncha</i>	<i>Cyrtorhyncha</i>	<i>Oxygraphis</i> ?	-	-
<i>Ficaria</i> Schaeff.	<i>R. subg. Ficaria</i>	<i>R. subg. Ficaria</i>	<i>R. sect. Ficaria</i>	<i>Ficaria</i>	<i>Ficaria</i>
<i>Halerpestes</i> Greene	<i>R. subg. R. sect. Halodes</i>	<i>Halerpestes</i>	<i>Oxygraphis</i>	<i>Halerpestes</i>	<i>Ranunculus</i>
<i>Hamadryas</i> Juss.	-	<i>Hamadryas</i>	-	-	<i>Anemoneae</i>
<i>Krapfia</i> DC.	-	<i>Krapfia</i>	-	-	<i>Ranunculus</i>
<i>Kumlienina</i> Greene	<i>R. subg. R. sect. Pseudaphanostemma</i>	<i>Kumlienina</i>	<i>Oxygraphis</i> ?	-	-
<i>Laccopetalum</i> Ulbr.	-	<i>Laccopetalum</i>	-	-	-
<i>Myosurus</i> L.	<i>Myosurus</i>	<i>Myosurus</i>	<i>Myosurus</i>	<i>Myosurus</i>	<i>Myosurus</i>
<i>Oxygraphis</i> Prantl	<i>R. subg. Oxygraphis</i>	<i>Oxygraphis</i>	<i>Oxygraphis</i>	<i>Oxygraphis</i> subg. <i>Euoxygraphis</i>	-
<i>Paroxygraphis</i> W. W. Sm.	-	<i>Paroxygraphis</i>	-	-	-
<i>Peltocalathos</i> Tamura	-	<i>Peltocalathos</i>	-	-	-
<i>Ranunculus</i> L.	<i>Ranunculus</i>	<i>Ranunculus</i>	<i>Ranunculus</i>	<i>Ranunculus</i>	<i>Ranunculus</i>
	-	<i>Aphanostemma</i>	<i>R. sect. Marsypadenium</i>		<i>Ranunculus</i>
	<i>R. subg. Batrachium</i>	<i>R. subg. Batrachium</i>	<i>R. sect. Marsypadenium</i>	<i>Batrachium</i>	<i>R. sect. Batrachium</i>
<i>Trautvetteria</i> Fisch. & C. A. Mey.	<i>Trautvetteria</i>	<i>Trautvetteria</i>	<i>Trautvetteria</i>	<i>Trautvetteria</i>	-

## RESULTS

**Molecular data.** -- Total sequence length for the ITS, *matK/trnK* and *psbJ-petA* regions in the 55 taxa are 595--617, 1543--1821 and 461--507 bp, respectively. We used 3416 aligned nucleotide positions in total: 650 bp in the ITS data set and 2766 bp in the chloroplast data set.

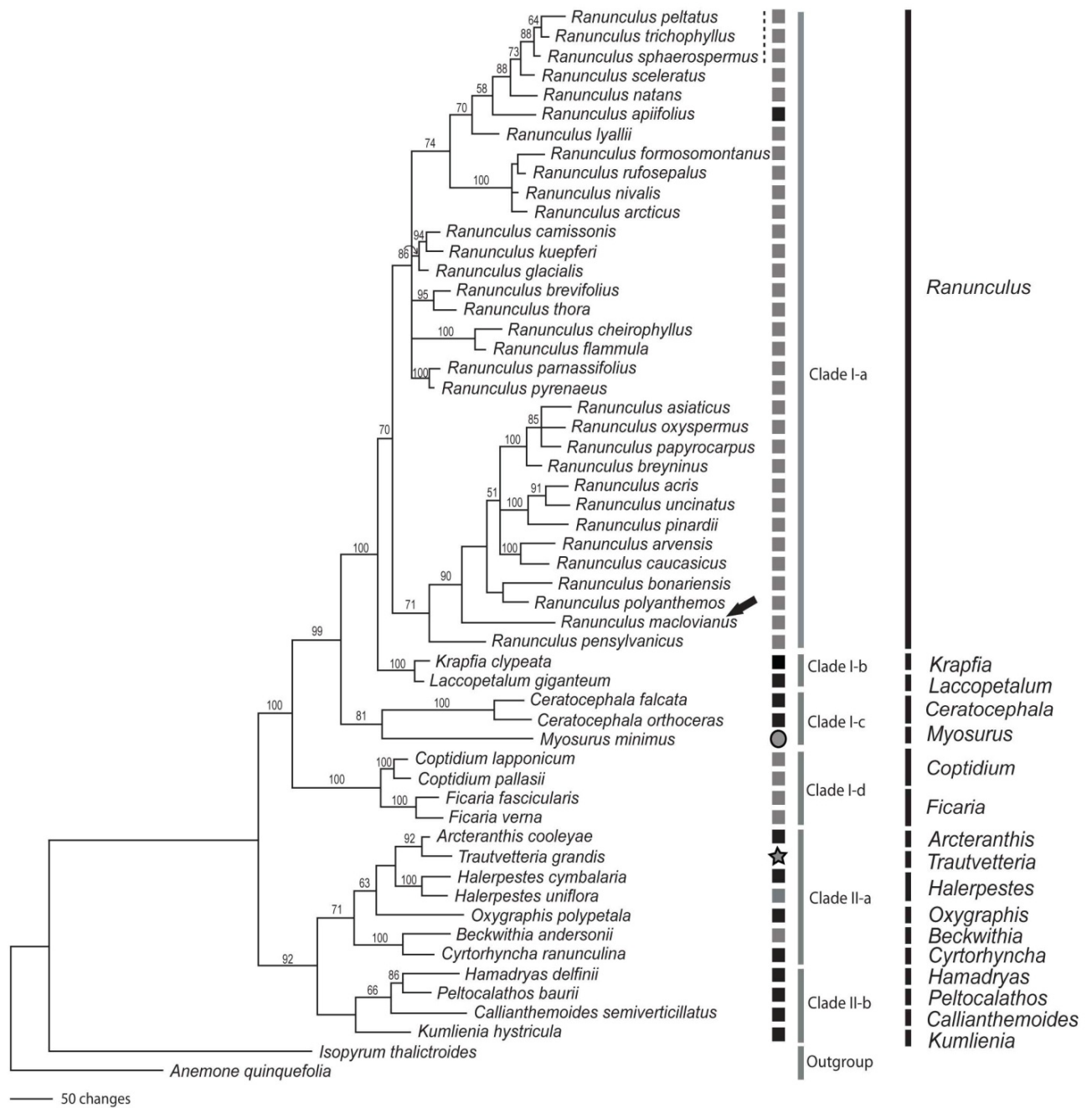
The analysis of the ITS data set resulted in 147 most parsimonious trees with a length of 1335 steps (252 parsimony informative characters, consistency index [CI] = 0.49, retention index [RI] = 0.61, rescaled consistency index [RC] = 0.30). In the strict consensus tree the *Myosurus-Ceratocephala* clade was found sister to a large clade of taxa with 66% bootstrap support. This large clade contained a polytomy with *Ranunculus*, *Arcteranthis*, *Beckwithia*, *Callianthemoides*, *Coptidium*, *Cyrtorhyncha*, *Ficaria*, *Halerpestes*, *Hamadryas*, *Kumlienina*, *Oxygraphis*, *Peltocalathos* and *Trautvetteria*. *Krapfia* and *Laccopetalum* formed a strongly

supported monophyletic group (100% BS) within *Ranunculus*, but their position in the core *Ranunculus* clade was weakly supported. The monotypic South American genus *Aphanostemma* also emerged within the core *Ranunculus* clade (*R. apiifolius*); (data not shown).

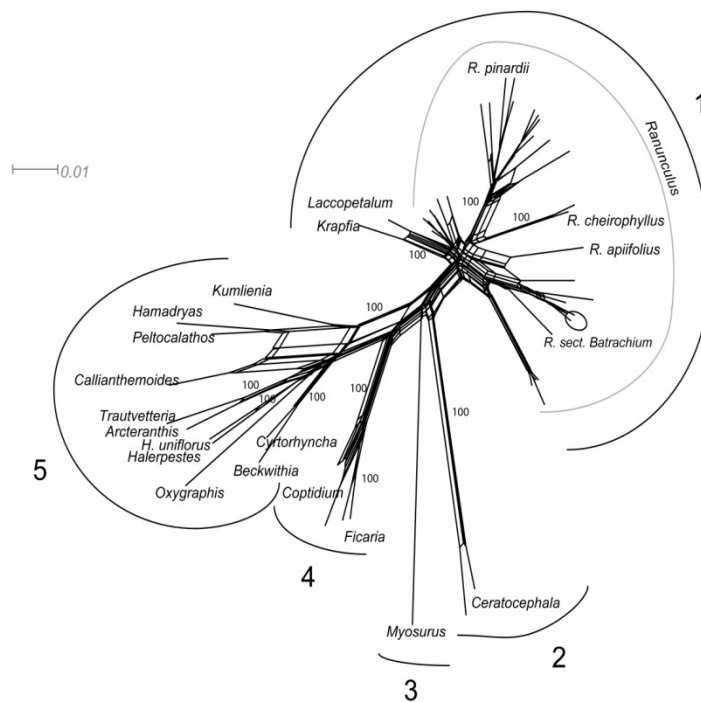
Analysis of the combined chloroplast data set (*matK/trnK* and *psbJ-petA*) resulted in 60 most parsimonious trees with a length of 2860 steps (787 parsimony informative characters, consistency index [CI] = 0.62, retention index [RI] = 0.73, rescaled consistency index [RC] = 0.46). The strict consensus tree contained two major clades with 100% bootstrap for each clade (not shown). The first clade comprised *Ceratocephala*, *Coptidium*, *Ficaria* and *Myosurus* and was sister to the core *Ranunculus* clade, *R. apiifolius* (*Aphanostemma*) was nested again within the core *Ranunculus* clade. *Krapfia* and *Laccopetalum* formed a monophyletic clade with high bootstrap support which was nested within the core *Ranunculus* clade with a low bootstrap support. The second clade also contained dichotomous split. *Arcteranthis*, *Halerpestes*, *Oxygraphis* and *Trautvetteria* formed a monophyletic group which was sister to clade IIb formed by *Callianthemoides*, *Hamadryas*, *Kumlienia* and *Peltocalathos*.

Parsimony analysis of the combined data set resulted in 33 most parsimonious trees of 4316 steps (1039 parsimony informative characters, consistency index [CI] = 0.56, retention index [RI] = 0.67, rescaled consistency index [RC] = 0.38). This tree showed greater resolution and more well-supported nodes than the trees from the datasets analyzed independently. The topology provided by the combined data is similar to the topology of the chloroplast data (Fig. 1), except for the position of the *Krapfia-Laccopetalum* clade which, in the combined analysis, was found sister to the core *Ranunculus* clade.

The Neighbor Net (NNet) analysis (Fig. 2), in which indels were not considered as informative characters, did not confirm the basic dichotomy of two major clades (clade I and II) found in the parsimony analysis. Instead, NNet identified five strongly supported splits (and clusters) which correspond partly to the well-supported clades in the topology of the combined tree obtained with the parsimony analysis (Fig. 2). The first cluster in the NNet splits graph comprised clade I-a and I-b in Fig. 1 and united the *Krapfia-Laccopetalum* group with *Ranunculus* s.str. Within *Ranunculus* s.str., the nesting of *R. pinardii*, *R. apiifolius*, *R. cheirophyllus* and *R. sect. Batrachium* within *Ranunculus* s.str. in the NNet splits graph is congruent with the results of the parsimony analysis (see clade 1a; Fig. 1).



**Fig. 1.** Strict consensus of 33 most parsimonious trees from the combined ITS, *matK/trnK* and *psbJ-petA* data set. Generic names correspond to accepted names in this study. Numbers listed above the horizontal lines are bootstrap values  $\geq 50\%$ . Signs represent the subtribes used in Tamura (1995) classification: Circle, *Myosurinae*; asterisk, *Trautvetteriinae*; squares, *Ranunculinae*, black squares are genera, gray squares species of *Ranunculus*. The arrow represents the position of the *Krapfia-Laccopetalum* clade in the topology based on chloroplast markers only. The dashed line indicates the clade corresponding to *R.* sect. *Batrachium*. The genus names in the right column indicate the finally accepted classification. For further synonyms, see Table 1.



**Fig. 2.** Neighbor Net (NNet) splits graph based on combined ITS, *matK/trnK* and *psbJ-petA* datasets. Clusters correspond to those well-supported clades shown in the topology of the combined tree. The first group corresponds to the clade I-a plus I-b, the second and third group are the same as clade I-c and the fourth cluster refer to clade I-d, and the last group corresponds to clades II-a and II-b. Bootstrap support values = 100 are shown.

The second cluster in the NNet splits graph comprised only *Ceratocephala* and there was little support evident in the splits graph analysis for a split that linked this genus with *Myosurus* (cluster 3). In contrast, parsimony analysis united these two clusters in clade 1c (Fig. 1). Cluster 4 comprised *Coptidium* and *Ficaria* and this inferred relationship is congruent with clade 1d (Fig. 1). Cluster 5 united the remainder of the Ranunculeae genera as found in the parsimony analyses; however, support for separation of species belonging to clade IIa and IIb within this cluster was less clear.

**Morphological data.** -- From the 33 morphological characters studied, only the structures of the achene surface suggested relationships among taxa congruent with the two main clades of the molecular tree. A sclerenchymatous layer in the pericarp of the achene (e.g., Fig. 3) occurs in all genera of clade I except for *Coptidium*, but is missing in clade II (Fig. 5A). The presence of longitudinal, parallel, straight veins on the surface of achenes occurs in most

genera of clade II (except for *Beckwithia*) but the venation pattern is specific for genera (Fig. 4, A-H): *Kumlienia* (Fig. 4B) and *Oxygraphis* (Fig. 4G) show only one big vein, the other genera (Figs. 4A, C, D, E, F, H) have more, but smaller longitudinal veins. The genera of clade I have no veins on the lateral surface (Figs. 4I to 4P) except for some species of *Ranunculus* s.str. which have irregular anastomosing or strongly curved veins on the lateral surface (Appendix 2).

*Ranunculus* species of the section *Batrachium* (Fig. 4P) have transversal ridges on the achene surface, which are formed by up-turned edges of elongated sclereid cells (Cook, 1963). *Ranunculus arvensis* (Fig. 4M), and *R. pinardii* (Fig. 4N) have spiny or tuberculate achenes which are formed by the parenchyma layer of the pericarp (Lonay, 1901). These macroscopic surface structures found in *Ranunculus* s.str. have a different anatomical background than the venation patterns.

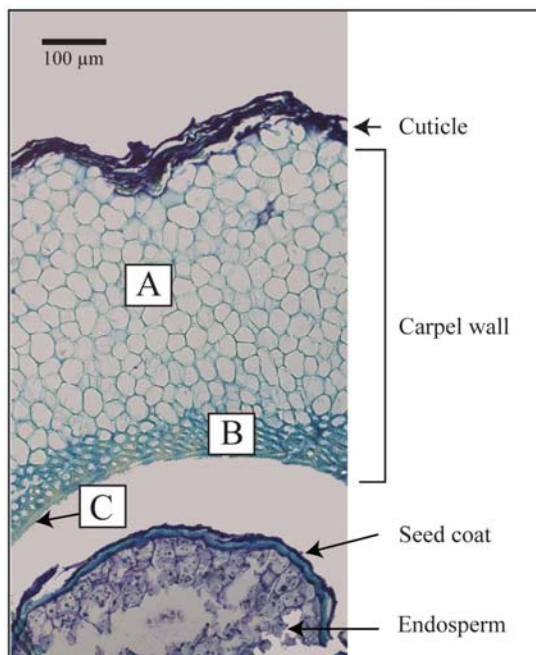
The microstructure of the surface of the pericarp surface is mostly irregular rugose (Fig. 4A-G), with pronounced convex cell surfaces in *Cyrtorhyncha* (Fig. 4H) and a reticulate cell pattern in *Myosurus* (Fig. 4J). Within *Ranunculus* s.str., finely papillate (Fig. 4M), foveolate (Fig. 4O) and rugose (Fig. 4P) microstructures are present.

Palynological studies identified seven types of apertures in the pollen grains (Fig. 5C). Diversity of aperture types was even observed at the species level as well, e.g. in *Beckwithia andersonii*, *Cyrtorhyncha ranunculina*, *Ficaria fascicularis*, and *Ranunculus pensylvanicus* (Fig. 5C). Mapping the character states on the phylogenetic tree based on the combined nuclear and chloroplast sequences suggested that the tricolpate type is ancestral in the tribe, but the variation of this character is too high to characterize genera. Only the *Krapfia-Laccopetalum* clade has a consistently periporate aperture type.

Morphological characters of the perianth, i. e. presence of petals or the shape of the nectary, are not congruent with the tree topology of the combined nuclear and chloroplast sequences (e.g., Fig. 4B). Other morphological characters are either unique for certain genera (Appendix 2) or show an overall high level of homoplasy.

**Table 2.** Character list and their corresponding states used in this study. Signs refer to the bibliographic source used, † Tamura, 1995; ‡ Goepfert, 1974. In the case no plant material or no complete vouchers were available, states were extracted from the literature cited in the materials and methods section.

Number	Character	Character States
1	Life form	(0) annual - biennial, (1) perennial
2	Number of flowers	(0) one, (1) more than one
3	Flower position	(0) terminal inflorescence, (1) axillary in stem leaves, (2) arising from basal rosette
4	Flower	(0) bisexual , (1) unisexual
5	Sepals	(0) sepaloid, (1) petaloid
6	Consistency of petal and sepal	(0) not fleshy , (1) fleshy
7	Number of sepals	(0) three, (1) four, (2) more than four
8	Presence of spur in the sepal	(0) absent , (1) present
9	Petals	(0) present, (1) absent
10	Number of petals	(0) less than five , (1) five ,(2) more than five, (-) not applicable
11	Color of petals	(0) yellow, (1) other than yellow, (-) not applicable
12	Shape of nectary	(0) ridge, (1) flap, (2) pocket, (3) U-form, (4) ring, (5) double scale, (-) not applicable
13	Number of nectary glands	(0) single, (1) three, (2) more than three
14	Androecium & gynoecium	(0) not separated (androgynophore), (1) separated
15	Indumentum of receptacle	(0) glabrous, (1) hairy
16	Shape of fruit (length/width ratio)	(0) globose (0.5 - 0.1), (1) ellipsoid (1.0 - 2.5), (2) elongated (2.5 -5), (3) linear (>5)
17	Ovule	(0) not pendulous, (1) pendulous
18	Connection of achenes	(0) connate, (1) not connate
19	Veins on achenes surface	(0) absent, (1) present, parallel & straight, (2) present, irregular reticulated & curved
20	Size of achenes (in mm)	(0) <1.5 , (1) 1.5-3.0, (2) 3.0-4.5, (3) >4.5
21	Shape of achenes	(0) compressed, (1) swollen, (2) swollen with lateral bulges, (3) triangular
22	Sclerenchyma layer of achenes†	(0) present, (1) absent
23	Spongy tissue of achenes†	(0) present, (1) absent
24	Achene surface (microstructure × 570)	(0) irregular rugose, (1) fine papillose, (2) foveolate, (3) reticulate rugose
25	Achene surface, tubercles or spines (macrostructure × 5)	(0) absent, (1) present
26	Achene surface, transversely wrinkles (macrostructure × 5)	(0) absent, (1) present
27	Indumentum of achenes	(0) glabrous, (1) partly hairy, (2) hairy throughout
28	Margin of achenes	(0) inconspicuous, (1) bordered, (2) winged
29	Stalk of achenes (mm)	(0) short or missing (up to 0.5 mm) (1) long (>0.5 mm)
30	Beak length	(0) equalling body of achene, (1) shorter than body of achene, (3) missing
31	Shape of beak ( length/ width)	(0) >5, (1) <1, (2) 1-5 , (3) missing
32	Basic chromosome number (X)‡	(0) 8, (1) 7
33	Pollen aperture type	(0) syncolpate, (1) dicolpate, (2) tricolpate, (3) stephanocolpate, (4) pericarpate, (5) periporate



**Fig. 3.** Cross section of a mature achene of *Ranunculus acaulis* and details of its structure. A, parenchymatous cells, B, inner part of carpel wall with thick-walled cells (sclerenchymatous layer), C, inner epidermal layer of the carpel wall.

## DISCUSSION

**Phylogenetic reconstruction and morphology.** -- Given the low resolution in the ITS topology, we have based the discussion of our results on analyses of the combined nuclear and chloroplast sequence datasets.

The parsimony analysis revealed a strict consensus tree with six main clades, clade I-a, -b, -c, -d and clade II-a, -b (Fig. 1) within the Ranunculeae. This grouping is incongruent with the classification of Tamura (1993, 1995) on subtribal level. Tamura subdivided Ranunculeae into three subtribes, *Trautvetteriinae*, *Myosurinae* and *Ranunculinae*. Under this classification, achenes in the *Trautvetteriinae* have no sclerenchymatous layer in the pericarp while in *Myosurinae* it is weakly developed. *Ranunculinae* on the other hand, have either well-developed sclerenchymatous layers or none at all. Clade 1 identified in our analysis includes mainly genera with a sclerenchymatous layer except *Coptidium* (Fig. 5A). Clade II includes *Trautvetteriinae* and the remaining genera of *Ranunculinae* sensu Tamura (Fig. 5A). Tamura's (1995) concept of subtribes is therefore not supported by the molecular data.

With the exception of some species of *Ranunculus*, the taxa in clade I have no distinct veins on the lateral surface of the achenes. However, the pattern of venation in these species is anastomosed or strongly curved and not longitudinal-parallel, as in the taxa of clade II. In the genera of clade I, veins occur only at the dorsal and ventral edges of the achenes (Lonay,

1901), which may be a derived character (Tamura, 1995). Although the sclerenchyma layer and venation patterns are not completely congruent with the molecular phylogeny, they represent probably the most conservative characters at the generic level. In their specific patterns, they can be used as diagnostic features.

Other macroscopic fruit structures might be best understood as dispersal mechanisms for certain taxa. For instance, *Ranunculus* section *Batrachium* has transversal ridges (Fig. 4P) which are formed by sclereid cells inside the pericarp (Cook, 1963). These ridges are breaking zones allowing the passage of water during germination. This feature could potentially be advantageous in aquatic habitats (Cook, 1963). Presence of spiny, tuberculate, and hooked structures of the pericarp such as in *Ranunculus arvensis* (Fig. 4M) and in *R. pinardii* (Fig. 4N) might be interpreted as an adaptation to epizoochory (Müller-Schneider, 1986). In *Ceratocephala* the achenes do not fall apart at maturity and the collective fruit, with its spine-like long beaks, is dispersed as a whole. All these taxa occur in dry areas, where spiny diaspores are an efficient dispersal mechanism via epizoochory.

Neither analyses of molecular nor morphological data revealed a strongly supported basal subdivision of the tribe. The basal dichotomy of the parsimony analysis (clades I and II) is not supported by a strong split in the Neighbor Net analysis, and this division is not supported by the presence/absence of any shared morphological characters. The Neighbor Net analysis does not suggest a strongly hierarchical (bifurcating) structure of the data, but rather indicates a network composed of five major lineages (Fig. 2). These graph features do not provide support for an hypothesis of gradual evolution (Hoot & al., 2008), as might be inferred from a bifurcating tree-topology. Rather they suggest that the main genetic lineages diverged within a relatively short geological time period, most likely within the Eocene (Paun & al., 2005; Hoffmann & al., 2010). The major lineages identified in the Neighbor Net analyses are not supported by morphological features and do not correspond to previous classifications, although as mentioned above, they correspond partly to well-supported clades of the parsimony analysis (Fig. 1). Greater congruence of molecular data and morphological characters occurs in the terminal clades. Presence of a lack of resolution in relationships of the some clades (e.g. clade II-a, II-b) is probably due to rapid ancient radiation, an inference consistent with the shape of the NNet splits graph (Fig. 2).

**Clade I-a: *Ranunculus* clade:** -- The maximum parsimony analysis revealed a well-supported main *Ranunculus* clade including *Aphanostemma* and subgenus *Batrachium* (Fig. 1), consistent with findings from earlier studies (Hörandl & al., 2005; Paun & al., 2005).

*Ranunculus pinardii*, which had been previously unstudied, is clearly nested within *Ranunculus*.

*Ranunculus apiifolius* was described by Persoon in 1806. However, later this species was assigned to a monotypic genus, *Aphanostemma* (Pers.) A. St.-Hil. (1825) in consideration of its small, bilabiate petals. In our tree topology, based on all markers, this species was nested within the core *Ranunculus* clade as in previous studies (Hörandl & al., 2005; Paun & al., 2005; Lehnebach & al., 2007). A morphology-based cladistic analysis placed *Aphanostemma apiifolius* in a clade with *Ranunculus* as well (Loconte & al., 1995). The very small petals in this species have been assumed to be an ancestral feature in the tribe (Janchen, 1949; Tamura, 1995). However, reduced petals occur in different genera within the tribe several times, e.g. in *Kumlienia hystricula* and, less pronounced, in *Arcteranthis cooleyae* (Fig. 5B). In Ranunculaceae, the formation of petals is probably controlled by a shared, homologous developmental program that can be rapidly modified by gene expression patterns (Rasmussen & al., 2009). Our character optimization suggests that the presence of petals is ancestral in Ranunculeae (Fig. 5B). In other genera with reduced petals, the large and colored petaloid sepals have an insect-attracting function. In *R. apiifolius* the sepals are also inconspicuous and the perianth may be secondarily reduced in this annual, ephemeral species. The surface of the achenes is similar to other *Ranunculus* species (Fig. 7K) and lacks longitudinal veins or other prominences on the surface of the achene. Considering the molecular evidence and the presence of only a single autapomorphy, the species should be kept as *Ranunculus apiifolius*.

In all our analyses, species of *R. sect. Batrachium* (*R. peltatus*, *R. trichophyllus*, *R. sphaerospermus*) form a distinct clade with high bootstrap support nested in the core *Ranunculus* clade, as sister to other species growing in wet habitats (Figs. 1, 2), e.g. *Ranunculus natans* (Hörandl & al., 2005; Paun & al., 2005; Gehrke & Linder, 2009; Hoffmann & al., 2010; Emadzade & al., unpubl.). *Batrachium* was described by Candolle (1817) as a section of *Ranunculus* and elevated to generic status by Gray (1821). This section includes aquatic species having white petals with no starch layer, reduced nectary pits, achenes with transversal ridges on the surface, and often heterophyllous leaves. The two latter characters are putative adaptations to aquatic habitats (Cook, 1963, 1966). Transverse ridges on the surface of the achene, which is one of most characteristic features of this group (Fig. 4L), occur also in some species of *Ranunculus*, e.g. *R. sceleratus*, *R. rivularis* (Cook, 1963). According to these morphological characters and molecular data, the classification of this group of species as a section of *Ranunculus* is supported (Hörandl, in press). All other representatives of sections of *Ranunculus* s.str. sensu Tamura, including *R. cheirophyllus* as a

member of *R.* sect. *Ficariifolius*, were nested within *Ranunculus* (Fig. 1). A more comprehensive discussion of sections within *Ranunculus* has been presented in Hörandl & al., 2005 and Hörandl, in press). A formal infrageneric classification of *Ranunculus* will be presented elsewhere (Hörandl & Emadzade, in prep.).

Our study also confirms the position of the former monotypic Central Asian genus *Gampsoceras* within *Ranunculus*. *Gampsoceras pinardii* was first described by Steven (1852) but later classified as a member of the genus *Ranunculus* in the subgenus *Gampsoceras* specifically by Tamura (1991, 1995). *Ranunculus pinardii* is an annual species with conspicuous flat, spiny and tuberculate fruits with very long, apically hooked beaks. The surface structure and the size of achenes resemble achenes of *R. arvensis*, and the length of the beaks is similar to those of *Ceratocephala* (Fig. 4N). The molecular tree based on the combined sequence data reveals that this taxon is nested within the *Ranunculus* clade, with a strongly supported sister relationship with the perennial species *R. uncinatus* and *R. acris* (Fig. 1). A more comprehensive phylogenetic analysis of *Ranunculus* s.str. places *R. pinardii* together with other species of the Irano-Turanian region (*R. sericeus*, *R. strigillosus*, and *R. constantinopolitanus*; Emadzade & al., subm.). Appendages on the diaspores increase the potential for epizoochorous dispersal (Tackenberg & al., 2006; de Pablos & Peco, 2007).

**Clade I-b, *Krapfia-Laccopetalum* clade.** – *Krapfia*, comprising eight species, and the monotypic genus *Laccopetalum* are endemic to the central Andes of South America. In all our analyses these two species form a strongly supported clade (Fig. 1, BS: 100). Previous molecular studies confirmed the sister relationship of the two genera (Lehnebach & al., 2007; Hoot & al., 2008). The tree topology based on combined chloroplast markers shows that this clade is nested in the core *Ranunculus* clade with low bootstrap support (its position is indicated in Fig. 1). The analyses based on combined nuclear and chloroplast markers placed this clade sister to the core *Ranunculus* clade without high bootstrap support. The *Krapfia-Laccopetalum* clade shows only 14 substitutions compared to the *Ranunculus* clade based on the combined data. In this case, more markers might be needed to resolve the position of these taxa. The Neighbor Net analysis places the *Krapfia-Laccopetalum* group within the core group of *Ranunculus* and indicates incompatibilities consistent with reticulate evolution in the *Krapfia-Laccopetalum* clade (Fig. 2). Lehnebach & al. (2007), analyzing *matK/trnK*, and Hoot & al. (2008), analyzing *atpB* and *rbcL*, found that the *Krapfia-Laccopetalum* clade was placed outside of the *Ranunculus* clade, but with low bootstrap support. In these studies,

species representing the genus *Ranunculus* were not available from all sections, so the position of the *Krapfia-Laccopetalum* clade was less reliable.

*Krapfia* and *Laccopetalum* have subglobose flowers, concave, thick sepals and petals, fleshy and clavate receptacles with both stamens and carpels attached (androgynophore), and finally a very distinct character, a free zone between the carpellate and the staminate areas. Both genera have numerous (in *Laccopetalum* up to 10,000) small carpels. Petals in *Krapfia* have one to three nectaries, whereas petals in *Laccopetalum* have many (up to 30) nectaries. These two genera can be distinguished by these characters from other taxa in Ranunculaceae (Tamura, 1995; Lehnebach & al., 2007). Multiple nectary glands occur in alpine *Ranunculus* species from New Zealand as well, but the position and shape differ from those of *Laccopetalum* (Lehnebach & al., 2007). Previous molecular studies have shown that the species from New Zealand are not related to the *Krapfia-Laccopetalum* clade (Lehnebach & al., 2007). Additional to these characters, our palynological study showed that *Krapfia* and *Laccopetalum* have pantoporate pollen grains which occur in other species of *Ranunculus*, as well (Fig. 5C; Santisuk, 1979). Pantoporate pollen does not occur in any other genus of the tribe except for *Coptidium* (*C. pallasii*). However, pores in *Coptidium* are elongate and represent an intermediate stage between a pantoporate and pantocolpate pattern. *Laccopetalum giganteum* has a special kind of pericolpate pollen which is not observed in other taxa. It has six relatively large pores, whereas other taxa have pollen grains smaller in size and with more than six pores. A morphology-based cladistic analysis has suggested that *Laccopetalum* and *Krapfia* are sister to *Ceratocephala*, but not to *Ranunculus* (Loconte & al., 1995).

We hypothesize that the strong geographical isolation of this clade in the Andes has resulted in the evolution of very distinct morphological characters in these genera. Fleshy sepals and receptacles and also coriaceous leaves could be adaptations to xerophytic conditions (Lehnebach & al., 2007). The molecular data suggest a close relationship of this clade to *Ranunculus* s.str. or even derivation from the core *Ranunculus* clade (Fig. 2). The two genera could be included into *Ranunculus* s.str., as suggested by Janchen (1949). Nevertheless, because of their combination of unique morphological characters, we will maintain these two species as members of separate genera. Future studies should include more species of *Krapfia* to test the phylogenetic placement of *Laccopetalum* and its relationship to *Krapfia*.

**Clade I-c, *Ceratocephala-Myosurus* clade.** -- The position of the *Ceratocephala-Myosurus* clade as sister to the core *Ranunculus* clade has been reported in all previous studies based on plastid and nuclear markers (Paun & al., 2005; Lehnebach & al., 2006, Hoot & al., 2008; Gehrke & Linder, 2009; Hoffmann & al., 2010). In our study, the maximum parsimony tree topology based on the combined dataset reveals that the *Ceratocephala-Myosurus* clade was sister to the *Ranunculus* clade with high bootstrap support (Fig. 1). *Myosurus* and *Ceratocephala* form one clade in the MP analysis, but do not cluster together in the Neighbor Net analysis (Fig. 2, cluster 2 & 3). The result of Neighbor Net analysis shows that these genera not only are separated from the core *Ranunculus* clade, but they are also highly diverged from each other.

The distinctive morphological characters of both *Ceratocephala* and *Myosurus* support their segregation from *Ranunculus*. The achenes of *Ceratocephala* have inflated empty chambers on either sides, an elongated beak, except one endemic species in New Zealand (Garnock-Jones, 1984), and a base chromosome number  $x = 7$ , which has been reported in only some species of *Ranunculus*. The karyotype of *Ceratocephala*, however, is different from these species (Goepfert, 1974). *Myosurus*, on the other hand, is a distinct small annual, scapose genus, distinguished from other genera of Ranunculeae by spurred sepals, strongly elongated fruits, a strong dorsal ridge on the achene (Fig. 4J) and pendant anatropous ovules. Other members of Ranunculeae have ascending hemitropous ovules (Tamura, 1995). *Myosurus*, which was described by Linnaeus (1753), has never been included in *Ranunculus* and was treated as the single member of *Myosurinae* by Tamura (1995). Chromosome studies in this taxon showed that chromosomes types are intermediate between the R-Type and the T-Type (Kurita, 1963), although T-type chromosomes have not been reported in Ranunculeae. Two shared morphological characters of *Ceratocephala* and *Myosurus* are the persistence of hypocotyl and the development of adventitious roots in the transitional zone between the hypocotyl and the primary root (Tamura, 1995). These features are also observed in *R. pinardii* and could play a role in the rapid development of root systems in annual species. Our SEM study shows that *Myosurus* has an unusual reticulate microstructure on the surface of the pericarp (Fig. 4D). This pattern has neither been observed in any other species of *Ranunculus* nor in allied genera.

These distinctive morphological and chromosomal characters, the molecular data and high sequence divergence in the *psbJ*–*petA* region provide strong support for the exclusion of *Ceratocephala* and *Myosurus* from *Ranunculus* as classified by most European and Asian authors (Ovczinnikov, 1937; Iranshahr & al., 1992; Tutin & Cook, 1993; Hörandl, in press).

**Clade I-d, *Ficaria-Coptidium* clade.** -- All data sets support a close relationship between *Ficaria* and *Coptidium*, which is in agreement with previous molecular studies (Johansson, 1998; Hörandl & al., 2005; Paun & al., 2005; Hoot & al., 2008; Gehrke & Linder, 2009; Hoffmann & al., 2010).

The position of *Ficaria* has always been controversial and it has been considered as a subgenus, a section of *Ranunculus* or a separate genus. Tamura (1995) classified it as a subgenus of *Ranunculus*. However, *Ficaria* has been accepted by many Asian botanists as a separate genus because of its distinct features: three sepals, more than five petals, and stalked but non-beaked achenes (Ovczinnikov, 1937; Iranshahr & al., 1992). Similarly, *Coptidium*, with two species (*C. lapponicus* and *C. pallasii*), is differentiated from other *Ranunculus* species by three sepals and achenes without a sclerenchymatous layer but with two separate parts, the upper part filled with spongy tissue and the lower part containing the seed. This feature probably helps in the dispersal of the seed by water, i.e. hydatochory (Tamura, 1995). *Coptidium* differs also by pocket-like nectary scales from *Ficaria*, which has flap-like nectary scales. In the most recent revision of Ranunculaceae (Tamura, 1995), *C. pallasii* and *C. lapponicus* were classified as subgenera of *Ranunculus* (*Pallasiantha* and *Coptidium* respectively), based on petal color and leaf shape. Both species have four acrocentric and four metacentric pairs of chromosomes per diploid set (Goepfert, 1974). Flovik (1936) reported that *C. lapponicus* has particularly large chromosomes in comparison with other related taxa. This diploid species hybridizes with tetraploid *C. pallasii* and the triploid hybrid (*R. × spitzbergensis*) combines the different chromosomes of the parents (Benson, 1948; Cody & al., 1988). The shared fruit characters, the sister-relationship in the phylogenetic reconstruction and the interspecific hybridization of these taxa supports their treatment as a single genus, *Coptidium*.

Furthermore, the Neighbor Net analysis (Fig. 2) and all tree topologies based on nuclear and chloroplast markers (Fig. 1) show a clear separation of the *Ficaria-Coptidium* clade from the core *Ranunculus* clade (there are c. 160 substitutions between the core *Ranunculus* clade and the *Ficaria-Coptidium* clade in the combined data set). Results by Hoot & al. (2008) also confirm the separation of *Ficaria* (*Ranunculus ficaria* in this paper) from the core *Ranunculus* clade based on *atpB* and *rbcL* markers. In Hoot & al. (2008), *Ficaria* is sister to *Hamadryas*, *Halerpestes* and *Trautvetteria*. In our analysis, *Ficaria* is more closely related to the *Ceratocephala-Myosurus-Ranunculus* clade than to the *Hamadryas-Halerpestes-Trautvetteria* clade. Based on all the morphological and molecular evidence, we conclude that *Ficaria* and

*Coptidium* should be treated as genera and not merged with *Ranunculus*, in accordance with many Eurasian authors (Ovczinnikov, 1937; Iranshahr & al., 1992; Hörandl, in press).

**Clade II.** -- Our combined analysis shows that *Trautvetteria*, *Halerpestes*, *Oxygraphis*, *Arcteranthus*, *Beckwithia* and *Cyrtorhyncha* form one clade (Clade II-a, Fig. 1) and *Hamadryas*, *Peltocalathos*, *Callianthemoides* and *Kumlienia* another one (Clade II-b, Fig. 1). However, this subdivision is only weakly supported (71 BS for clade IIa) and is neither confirmed by Neighbor Net analysis, nor by earlier studies with an incomplete sampling of the tribe. In Hoot & al. (2008), *Oxygraphis* is sister to a clade comprising *Hamadryas*, *Peltocalathos*, and *Callianthemoides*. These two clades form a single strongly supported cluster in the Neighbor Net analysis (Fig. 2, 100% BS). In this analysis the two clades II-a and II-b are basal sister groups that are poorly resolved. Since the taxa in Clade II show mostly disjunct geographical distributions, and may have diverged between the Miocene and the early Pliocene (Paun & al., 2005, Emadzade & al., submitted), it is unlikely that the lack of resolution and incompatibilities visualized in the splitsgraph are the result of recent hybridization. A possible explanation is ancient radiation within clade II and a strong morphological divergence of taxa in different geographical areas.

**Clade II-a, *Arcteranthus*, *Beckwithia*, *Cyrtorhyncha*, *Halerpestes*, *Oxygraphis* and *Trautvetteria* clade.** -- *Oxygraphis*, a genus of four species, is located on a long branch in clade II-a and similarly so in all analyses (Figs. 1, 2). This taxon, with persistent and enlarged sepals, has been accepted by most taxonomists as a separate genus. Gray (1886) has emphasized that the texture of the carpels of *Oxygraphis* is so distinct that this taxon should be without any doubt excluded from *Ranunculus*. One of the main diagnostic characters of *Oxygraphis* is the persistence of sepals at the fruiting stage, although this character has also been observed in *Paroxygraphis*, *Beckwithia*, and *Ranunculus glacialis* as well. Similar to *O. polypetala*, all these taxa are distributed in high alpine zones or in the Arctic, and this feature might be explained as a homoplasious adaptive character that protects the fruits from wind or low temperatures in harsh cold climates. There is at least one longitudinal prominence on the surface of the achene (Fig. 4G). This feature, along with a small triangular beak, can be used to distinguish the achene of *Oxygraphis* from all other genera (Fig. 4G). In the maximum parsimony analysis, *Oxygraphis* represents a highly diverged lineage nested within clade II-a. Its phylogenetic placement within this clade is also supported by the Neighbor Net analysis (Fig. 2).

*Halerpestes* comprises about ten species and is relatively widespread in the northern hemisphere and in South America. The taxonomic status of *Halerpestes* has varied from being considered as a subgenus or section of *Ranunculus* to being included in *Oxygraphis* or treated as a separate genus. Due to the basic karyotype of four acrocentric and four metacentric chromosome pairs, Goepfert (1974) assumed that *Oxygraphis* has an ancestral status. Ploidy levels in *Halerpestes* vary from diploid to hexaploid. The tree topology based on combined data (Fig. 1) revealed its position in clade II-a, which agrees with the study by Hoot & al. (2008), which reported analyses of *atpB* and *rbcL* data. Our SEM studies on two species show that *Halerpestes* has flap-like nectary scales, in contrast to Tamura (1995), who described them as pocket-like. Variation of the nectary scale has been observed in *Ranunculus* s.str. as well.

Analyses of all molecular datasets suggest that *Halerpestes uniflora* is sister to *Halerpestes cymbalaria* with 100% bootstrap support (Figs. 1, 2). This species was described as *Ranunculus uniflorus* Phil. ex Reiche, endemic to the alpine zones of South America. It is a perennial species with entire leaves, three sepals, seven petals, and a high number of carpels (ca. 100). The presence of longitudinal veins on the achenes, which is typical of clade II, is similarly observed on the achenes of *H. uniflora* (Fig. 4J). The SEM study shows that the pattern of veins in this taxon is the same as in *H. cymbalaria* (Fig. 4I, J). Additionally, this taxon has tricolpate pollen as *H. cymbalaria*. According to these morphological characters, habitat and molecular data, we classify this species as a member of *Halerpestes*.

*Trautvetteria* has been treated as a single genus in *Trautvetteriinae* due to its apetalous by Tamura (1967). Tamura (1995) considered this genus as the ancestor of the whole tribe since monochlamydeous flowers have been considered as a primitive condition in the family. However, recent phylogenetic studies revealed the evolution of perianth differentiation for Ranunculales as highly dynamic; the condition of two perianth whorls, with the outer one sepaloid, the inner one tepaloid, is ancestral for Ranunculales, while the presence of petals and sepals is derived (Endress & Doyle, 2009). For the core Ranunculaceae, the ancestral state is that both tepal whorls are petaloid (Endress & Doyle, 2009). In Ranunculeae the presence of petals is the ancestral state (Fig. 5B). According to the scattered presence of some taxa with more or less reduced petals in the whole tree (*Ranunculus apiifolius*, *Kumlienia*, *Trautvetteria*), it is likely that apetalous flowers are a homoplasious, derived feature in this tribe (Fig. 5B). However, in all analyses *Trautvetteria* is nested in the clade II-a, sister to *Arcteranthis* with high bootstrap support. Previous studies based on *atpB* and *rbcL* provide

good support for the close relationship to *Hamadryas*, *Halerpestes*, and *Ficaria* in one clade with 60% BS (Hoot & al., 2008).

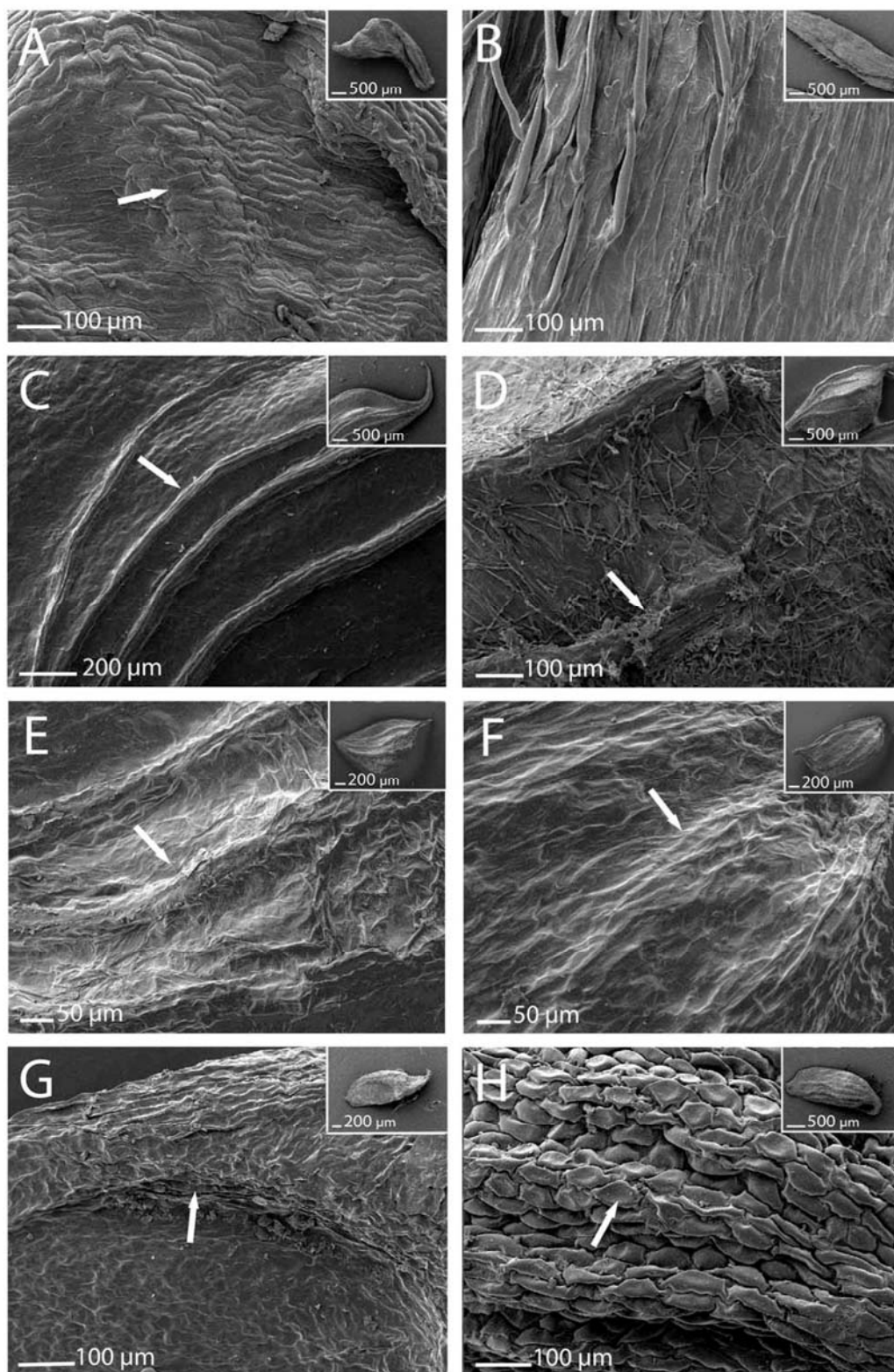
*Arcteranthus* and *Cyrtorhyncha* are monotypic genera endemic to northwestern and western North America, respectively. Based on the combined data, *Arcteranthus* shows a well-supported close relationship with *Trautvetteria* (Fig. 1). The Neighbor Net analysis also confirms this affinity (Fig. 2). *Trautvetteria* and *Arcteranthus* have a similar pattern in the veins on the surface of the achenes but *Trautvetteria* has some thin veins between the main veins which are lacking in *Arcteranthus* (Fig. 4C, D). Analyses of molecular data, reduced petals and petaloid sepals, and a partly shared distribution area in N. America strongly suggest a common ancestry of *Arcteranthus* and *Trautvetteria*.

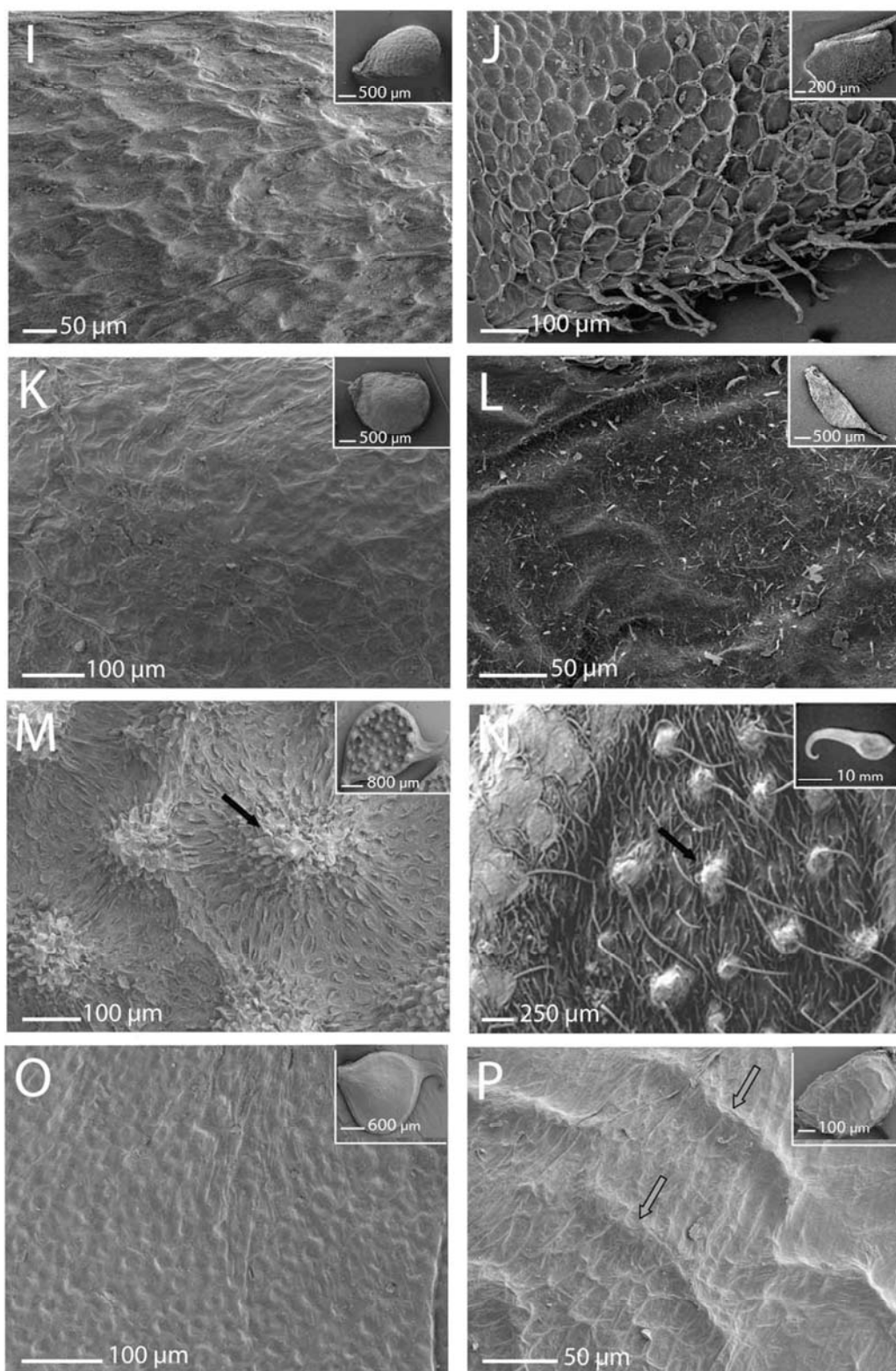
*Beckwithia andersonii*, which has been classified in *Ranunculus* subgen. *Crymodes* by Tamura (1967), is sister to *Cyrtorhyncha* and located in clade II-a in all our analyses (Figs. 1, 2). This genus is characterized by bladder-like fruitlets and membranaceous pericarps. Due to these characters some authors have described the fruit of this taxon as utricle (Whittemore, 1997). Membranaceous pericarps are observed in *Ranunculus papyrocarpus* as well. A cavity in the fruit could be some kind of adaptation to wind dispersal (Müller-Schneider, 1986).

Achenes of *Cyrtorhyncha* have long triangular hooked beaks and almost parallel longitudinal veins (Fig. 4H) which are unique within the tribe. Although there are no obvious morphological synapomorphic characters shared between *Cyrtorhyncha ranunculina* and *Beckwithia andersonii*, these two taxa form a clade with 100% BS in tree topologies based on combined nuclear and chloroplast data (Figs. 1, 2) and have a similar distribution area (Fig. 7).

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**Fig. 4** (next pages). Morphology of the achene surface of genera in tribe Ranunculeae, SEM micrographs. Small inserts show an overall view of the achene. A-H, taxa with longitudinal veins on the lateral surface (white arrows); I-P, taxa without longitudinal veins, but sometimes with tubercules (M-N, black arrow) or transversal ridges (P, grey arrow). **A**, *Hamadryas delfinii*; **B**, *Kumlienia hystriola*; **C**, *Arcteranthus cooleyae*; **D**, *Trautvetteria carolinensis*; **E**, *Halerpestes cymbalaria*; **F**, *H. uniflora*; **G**, *Oxygraphis polypetala*; **H**, *Cyrtorhyncha ranunculina*; **I**, *Ficaria fascicularis*; **J**, *Myosurus minimus*; **K**, *Ranunculus apiifolius*; **L**, *Coptidium pallasii*; **M**, *Ranunculus arvensis*; **N**, *R. pinardii*; **O**, *R. lanuginosus*; **P**, *R. trichophyllus*. The microstructure of the surface of the pericarp is described in the text.

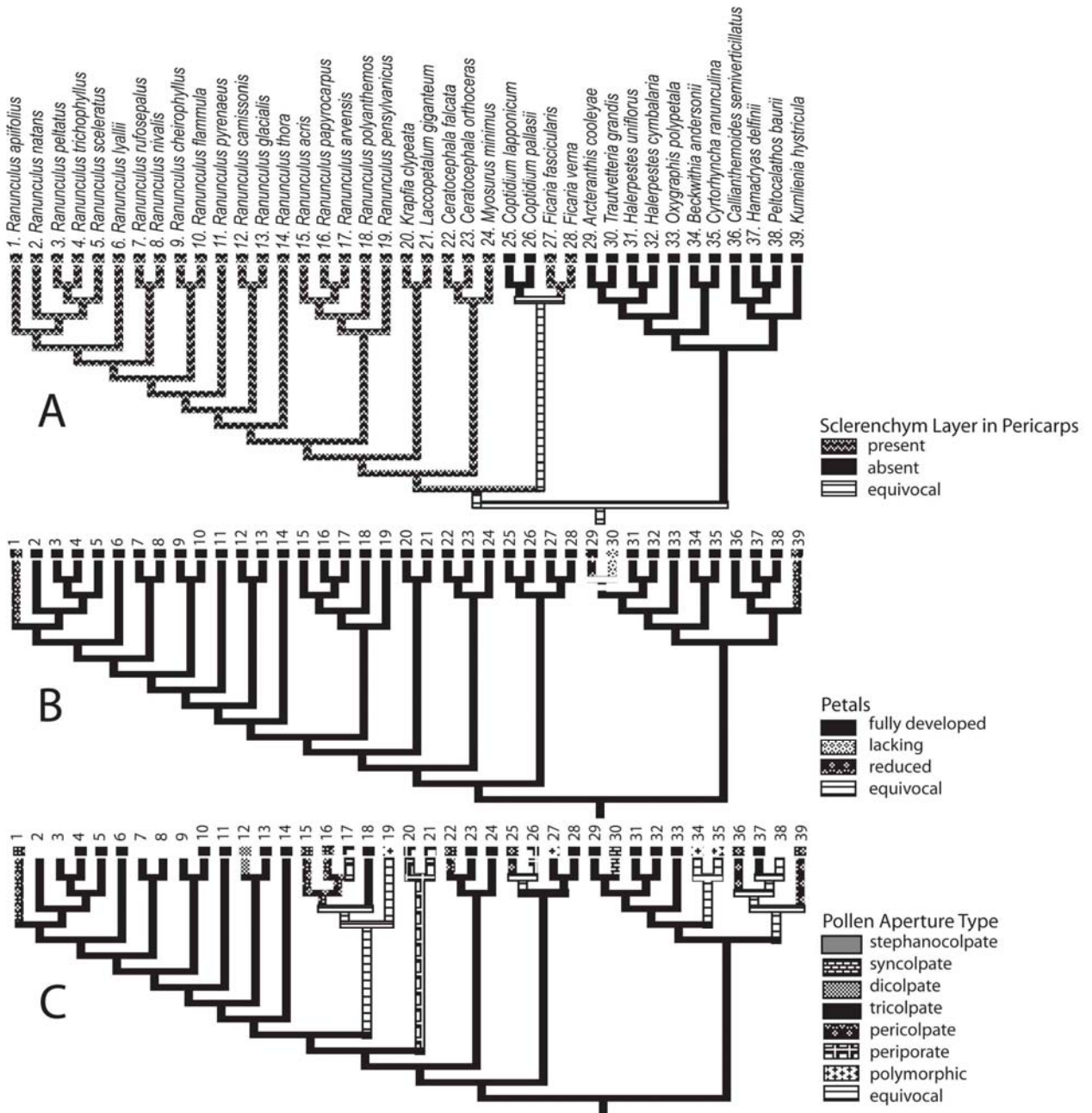




**Clade II–b, *Hamadryas*, *Peltocalathos*, *Callianthemoides* and *Kumlienia* clade.** -- *Hamadryas* is one of two dioecious genera in Ranunculeae (in addition to *Paroxygraphis*), and it is endemic to South America. Based on chloroplast data, it forms a clade with *Peltocalathos*, *Callianthemoides* and *Kumlienia*, but without high bootstrap support. This weakly supported and heterogeneous clade comprises four monotypic genera with distinct geographical distributions: *Hamadryas* and *Callianthemoides* are endemic to South America, *Peltocalathos* is endemic to South Africa, and *Kumlienia* is endemic to southwestern North America. All members of this clade have colored sepals. The main diagnostic characters of the members of this clade are: *Hamadryas* is dioecious; *Callianthemoides* has four to seven times pinnately ternate leaves; *Peltocalathos* has peltate, rounded leaves, and *Kumlienia* has small, cup-shaped petals and conspicuous white sepals. Our palynological study shows that *Callianthemoides semiverticillatus* and *Kumlienia hystricula* have pericolpate pollen while *Hamadryas delfinii* has tricolpate pollen. All of the species in this clade have pocket-like nectary scales except *Callianthemoides* which has a thickened nectary with a short scale. Each genus has a distinct shape of achenes. *Kumlienia* and *Peltocalathos* have elongated achenes, hairy in *Kumlienia* (Fig. 4B). Achenes are obovoid in *Callianthemoides* and semiovoid in *Hamadryas*. All of these four genera have distinct venation patterns on the surface of achenes (Fig. 4A, B). The morphological divergence in the clade is not accompanied by a pronounced genetic divergence, as inferred from branch lengths and relationships in the Neighbor Net analysis (Figs. 1, 2). The evolution of distinct morphological features is probably the result of a strong geographical isolation and rapid character evolution in different areas.

## TAXONOMIC CONCLUSIONS

Parallel, adaptive, and convergent evolution of morphological characters has occurred not only in *Ranunculus* and allied genera, but also the other genera of Ranunculaceae (Hoot, 1991; Hoot & al., 1994; Johansson, 1995, 1998; Ro & al., 1999; Lockhart & al., 2001; Hörandl & al., 2005; Paun & al., 2005). In fact, homoplasy of morphological characters has made morphology-based classifications in this tribe difficult. The molecular phylogenetic study provides the basic framework for an improved classification and a better understanding of character evolution.



**Fig. 5.** Optimization of four morphological characters on the tree topology based on the combined ITS, *matK/trnK* and *psbJ-petA* data set. **A**, sclerenchyma layer in the pericarp; **B**, presence of petals; **C**, Aperture type of pollen.

Most of the micro- and macromorphological characters studied here show incongruence with the molecular tree (Fig. 5). Our study suggests that fruit characters may be linked to dispersal mechanisms (e.g., achenes with spines, long hooked beaks, swollen fruits). The shape of the pollen apertures also shows parallel evolution. The basic and most common type

is tricolpate, which is observed in most of the *Ranunculus* species. Pollen aperture types probably have developed as an adaptation to certain pollinators or habitats (Proctor & al., 1996; Hesse, 2000; Tanaka & al., 2004). Characters of the perianth are probably based on a shared developmental program, and may be highly dynamic according to activation or de-activation of gene expression patterns (Rasmussen & al., 2009).

Our study confirms a great diversity of morphological characters which have evolved multiple times within the tribe. According to these characters and molecular studies, aggregating all genera of the tribe under *Ranunculus* s.l. would give a very heterogeneous taxon lacking common morphological features. Except for *Myosurus* and *Ceratocephala*, the morphological divergence in the clade is not accompanied by a pronounced genetic divergence, as inferred from branch lengths and genetic relationships as suggested in the Parsimony and Neighbor Net analyses (Figs. 1, 2). The Neighbour Net analysis confirmed that the genetic structure of the tribe is not hierarchical, but rather suggests several distinct clusters emerged out of an unresolved backbone phylogeny. Moreover, the two clades I and II each lack diagnostic morphological features. We agree with most authors that morphology is of crucial importance for a delimitation of genera (e.g., Stuessy, 2009 and literature therein), and we prefer to separate genera according to those well-supported clades or branches which can be identified by morphological features. These diagnostic characters can be used for identification. This concept fits largely to Tamura's (1995) narrow circumscription of genera, but avoids a polyphyletic genus *Ranunculus* s.str. by excluding *Ficaria* and *Coptidium*. We do not regard the morphological and genetic divergence of *R. apiifolius* as strong enough for a monotypic genus which would leave *Ranunculus* s.str. as a paraphyletic taxon (see discussion in Hörandl, 2006; 2007; Stuessy & König, 2008). Moreover, our data support acceptance of several monotypic genera in clade II, because none of the groupings suggested by the molecular data would be accompanied by shared morphological features. The strong geographical isolation of sister taxa over long time periods (e.g., in clade II-b) might have triggered the evolution of distinct, unique features, and further supports a delimitation of genera (e.g., Stuessy, 2009). The monotypic taxa could be relictual survivors of ancient radiations, or alternatively, they may have never diversified.

## TAXONOMIC IMPLICATIONS

We list here accepted generic names with their types and the most important synonyms, and new combinations. A full synonymy list for each taxon is available in Tamura (1995).

Tribe Ranunculeae DC.

**Arcteranthis** Greene, Pittonia 3: 190. 1897 – Type: *Ranunculus cooleyae* Vasey & Rose, Bull. Torrey Bot. Club. 19: 239. 1892.

**Beckwithia** Jeps., Erythea 6: 97. 1898 – Type: *Ranunculus andersonii* A.Gray, Proc. Amer. Acad. Arts 7: 327. 1868.

**Callianthemoides** Tamura, Acta Phytotax. Geobot. 43: 140. 1992 – Type: *Ranunculus semiverticillatus* Phil., Anales Univ. Chile 1: 60. 1861.

**Ceratocephala** Moench, Methodus (Moench) 218. 1794 – Type: *Ranunculus falcatus* L., Sp. Pl. 1: 556. 1753.

**Coptidium** Beurl. ex Rydb., Prodr. Stirp. Chap. Allerton Pp. 302. 1917 – Type: *Ranunculus lapponicus* L., Sp. Pl. 1: 553. 1753

**Cyrtorhyncha** Nutt. ex. Torr. & A. Gray, Fl. N. Amer. 1: 26. 1838 – Type: *Cyrtorhyncha ranunculina* Nutt. ex. Torr. & A. Gray.

**Ficaria** Guett., Hist. Acad. Roy. Sci. Mem. Math. Phys. 1750: 355. 1754 – Type: *Ranunculus ficaria* L., Sp. Pl. 1: 550. 1753.

**Halerpestes** E. Greene, Pittonia 4: 207. 1900 – Type: *Ranunculus cymbalaria* Pursh, Fl. Amer. Sept. (Pursh) 2: 392. 1814.

According to our results, a new combination is needed for *Halerpestes uniflora*:

**Halerpestes uniflora** (Phil. ex Reiche) Emadzade, Lehnebach, Lockhart & Hörandl, **comb. nov.** Basionym: *Ranunculus uniflorus* Phil. ex Reiche, Flora de Chile, 1: 16. 1896.

**Hamadryas** Comm. ex Juss., Gen. Pl. [Jussieu] 232. 1789 – Type: *Hamadryas magellanica* Lam., Encycl. (Lamarck) 3: 67. 1789.

**Krapfia** DC., Syst. Nat. (Candolle) 1: 228. 1817 – Type: *Krapfia ranunculina* DC.

**Kumlienia** E. Greene, Bull. Calif. Acad. Sci. 1: 337. 1886 – Type: *Kumlienia hystricula* (A. Gray) E. Greene, Bull. Calif. Acad. Sci. 1: 337. 1886.

**Laccopetalum** Ulbr., Bot. Jahrb. Syst. 37: 404. 1906 – Type: *Ranunculus giganteus* Wedd., Chlor. Andina, 2: 304. 1857.

**Myosurus** L., Sp. Pl. 1: 284. 1753 – Type: *M. minimus* L.

**Oxygraphis** Bunge, Verz. Altai. Pfl. 2: 46. 1836 – Type: *Ficaria glacialis* Fisch. ex DC. Prodr. (DC.) 1: 44. 1824.

**Paroxygraphis** W.W.Sm., Rec. Bot. Surv. India 4: 344. 1913 – Type: *Paroxygraphis sikkimensis* W.W.Sm.

***Peltocalathos*** Tamura, Acta Phytotax. Geobot. 43: 139. 1992 – Type: *Ranunculus baurii* Mac Owan, J. Linn. Soc. Bot. 18: 390. 1881.

***Ranunculus*** L., Sp. Pl. 1: 548. 1753 – Type: *Ranunculus acris* L., Sp. Pl. 2 1753 (Jarvis, 2007). Incl. *Batrachium* (DC.) S. F. Gray 1821; incl. *Aphanostemma* A. St. Hil. 1825; incl. *Gampsoceras* Steven 1852.

***Trautvetteria*** Fisch. & C. A. Mey., Index Seminum [St. Petersburg] 1: 22. 1835 – Type: *Cimicifuga palmata* Michx., Fl. Bor.-Amer. (Michaux) 1: 316. 1803.

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## Appendix 1. Materials used in this study (BG. Botanical garden)

Taxon (Synonym); Country; Collector, Collection number, Herbarium; ITS Genbank no.; *matK*/ *trnK* GenBank no.; *psbJ-petA* GenBank no.

*Anemone quinquefolia* L.; Connecticut; *Mehrhoff 12602* CONN; GU257978; GU257980; GU257995. *Arcteranthus cooleyae* (Vasey & Rose) Greene (*R. cooleyae*); Canada; *U. Jensen 28432* MPN; AY680201; -; GU258002. *Beckwithia andersonii* (A. Gray) Jeps. (*R. andersonii*); cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680197; AY954238; GU258003. *Callianthemoides semiverticillatus* (Philippi) Tamura (*R. semiverticillatus*); Argentina; *C. Lehnebach s.n.* VALD; AY680199; AY954236; Gothenburg, BG; *J.T. Johansson s.n.*; GU258004. *Ceratocephala falcata* (L.) Pers. (*R. falcatus*); Iran; *K.H. Rechinger, Jr. 50857* W; AY680191; AY954229; GU257996. *C. orthoceras* DC. (*R. testiculatus*); Austria; *E. Hörandl 3837* WU; AY680190; AY954230; GU257997. *Coptidium lapponicum* (L.) Tzvelev (*R. lapponicus*); Sweden; *J.T. Johansson s.n.* -; AY680194; AY954234; GU257998. *C. pallasii* (Schlecht.) Tzvelev (*R. pallasii*); Alaska; *R. Elven & al. SUP02-175* O; AY680195; AY954233; GU257999. *Cyrthorhyncha ranunculina* Nutt. ex Torr. & A. Gray. (*R. ranunculinus*); USA; *S. Nunn 1775* RM; GU257973; GU257981; GU258005. *Ficaria fascicularis* K.Koch (*R. kochii*); cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680193; AY954231; GU258000. *F. verna* Huds. ssp. *verna* (*R. ficaria* ssp. *bulbilifer*); Sweden; *J.T. Johansson s.n.* -; AY680192; AY954232; GU258001. *Halerpestes cymbalaria* (Pursh) Greene (*R. cymbalaria*); cult. Rezia BG; *J.T. Johansson 204* LD; AY680196; AY954237; GU258006. *H. uniflora* (Phil. ex. Reiche) Emadzade et al. (*R. uniflorus*); Chile; *C. Lehnebach s.n.* MPN; GU552270; GU552273; Argentina; *M. Weigend 7003* M; GU258007. *Hamadryas delfinii*; Argentina; *P. Schönschwetter AR08-20* WU; GU257974; GU257982; GU258011. *Isopyrum thalictroides* L.; Austria; *E. Hörandl 641* WU; GU257977; GU257979; GU258014. *Krapfia clypeata* (Ulbr.) Standl. & J.F. Macbr. (*R. clypeata*); Peru; *Sanchez & al. 11173* F, CPUN, MPN; GU552271; DQ490058; -. *Kumlienia hystricula* (A.Gray) E. Greene; USA; *E. Hörandl 9648* WU; GU257975; GU257983; GU258008. *Laccopetalum giganteum* Ulbr. (*R. giganteus*); Halle, BG; *J.T. Johansson s.n.*; GU552272; Peru; *Cano & al. 15196* USM; DQ400695; Halle, BG; *J.T. Johansson s.n.*; GU258009. *Myosurus minimus* L.; Genbank; AJ347913; AJ414344; -. *Oxygraphis polypetala* Hook. F. & Thomson; Nepal; ? 1926-3 LI; GU257976; GU257984; GU258012. *Peltocalathos baurii* (McOwan) Tamura (*R. baurii*); South Africa; *L. Mucina 030103/22* WU; AY680200; AY954235; GU258010. *Ranunculus acris* L.; cult. Bonn BG; *J.T. Johansson 194* CONN; AY680167; AY954199; GU258015. *R. apiifolius* Pers. (*Aphanostemma apiifolia*); Chile; *C. Lehnebach s.n.* VALD; AY680092; AY954140; Uruguay; *Lorentz 533* W; GU258016. *R. arvensis* L.; cult. Kiel BG; *J.T. Johansson 180* CONN; AY680177; AY954193; Iran; *Emadzade 109* WU; GU258017. *R. asiaticus* L.; Iran; *Shooshtari 2569* TARI; GU257963; GU257985; GU258018. *R. bonariensis* Poir.; Argentina; *P. Schönschwetter AR08-2a* WU; GU257964; GU257986; GU258019. *R. brevifolius* ssp. *brevifolius* Ten.; cult. Gothenburg BG; *J.T. Johansson s.n.* GB; AY680187; AY954212; GU258020. *R. breyninus* Cr. (*R. oreophilus*); Austria (loc. class.); *E. Hörandl 5249* WU; AY680115; AY954172; GU258021. *R. camissonis* Aucl. (*Beckwithia camissonis*); U.S.S.R.; *R. Koropewa s.n.* W; AY680083; AY954218; GU258022. *R. caucasicus* MB.; Georgia; *E. Hörandl 8259* WU; AY680178; AY954192;

GU258023. *R. cheirophyllum* Hayata; Taiwan; *E. Hörandl* 9550 WU; GU257965; GU257987; GU258024. *R. flammula* L.; cult. Oldenburg BG; *J.T. Johansson* 193 CONN; AY680185; AY954204; GU258025. *R. formosomontanus* Ohwi; Taiwan; *E. Hörandl* 9548 WU; GU257966; GU257988; GU258026. *R. glacialis* L.; Sweden; *J.T. Johansson s.n.* -; AY680082; AY954219; GU258027. *R. kuepferi ssp.orientalis* W. Huber; Austria; *E. Hörandl* 4336 WU; AY680085; AY954213; GU258028. *R. lyallii* Hook. f.; New Zealand; *M.A. Steel* 24603 MPN; AF323277; AY954142; *G. Schneeweiss & al.* - WU; GU258029. *R. maclovianus* Urv.; Chile; *C. Lehnebach s.n.* VALD; AY680158; AY954181; Argentina; *P. Schönschwetter* AR08-17 WU; GU258030. *R. natans* C.A.Mey.; Russia; *A. Tribsch* 9558 WU; AY680113; AY954134; GU258031. *R. nivalis* L.; Sweden; *J.T. Johansson s.n.*; AY680046; AY954123; GU258032. *R. oxyspermus* Willd.; Iran; *Emadzade* 100 WU; GU257967; GU257989; GU258033. *R. papyrocarpus* Rech. F., Aell. & Esfand.; Iran; *Tajeddini* 110 WU; GU257968; GU257990; GU258034. *R. parnassifolius ssp. parnassifolius* L.; France/Spain; *G. Schneeweiss & al.* 6509WU; AY680072; AY954224; GU258035. *R. pedatifidus* J.E. Smith, USA; *R. Orthner* 593RM; GU257969; GU257991; GU258036. *R. peltatus ssp. peltatus* Moench (*Batrachium peltatum*); cult. Nantes BG; *J.T. Johansson* 206 LD; AY680068; AY954131; GU258037. *R. pensylvanicus* L. f.; U.S.A.; *V. Zila* 447002 LI; AY680147; AY954190; GU258038. *R. pinardii* (Stev.) Boiss.; Iran; *Ghahremanii* 108 WU; GU257970; GU257992; GU258039. *R. polyanthemos* L.; Austria; *E. Hörandl* 5130 WU; AY680121; AY954185; GU258040. *R. pyrenaeus* L.; Spain; *G. Schneeweiss & al.* 6498 WU; AY680074; AY954225; GU258041. *R. rufosepalus* Franch.; Pakistan; *A. Millinger* 392897 LI; AY680047; AY954121; GU258042. *R. sceleratus* L.; Iran; *Emadzade* 112 WU; GU257971; GU257993; GU258043. *R. sphaerospermus* Boiss. & Blanche (*Batrachium sphaerospermum*); Turkey; *G. Dahlgren* B87B LD; AY680066; AY954132; GU258044. *R. thora* L.; cult. Lund BG; *J.T. Johansson* 223 LD; AY680188; AY954210; GU258045. *R. trichophyllum* Chaix (*Batrachium trichophyllum*); Greece; *G. Dahlgren* B23 LD; AY680067; AY954133; GU258046. *R. uncinatus* D. Don.; USA; *N. Holmgren* 5379 ZT; GU257972; GU257994; GU258047. *Trautvetteria grandis* Honda; cult. California BG; *J.T. Johansson* 82.1322 -; AY680202; AF007945; GU258013.

**Appendix 2.** Data matrix of 33 morphological characters of Ranunculeae. Inapplicable characters are coded with “-” and missing data are coded with “?”. Bold numbers (in the box) indicate autapomorphies at generic level.

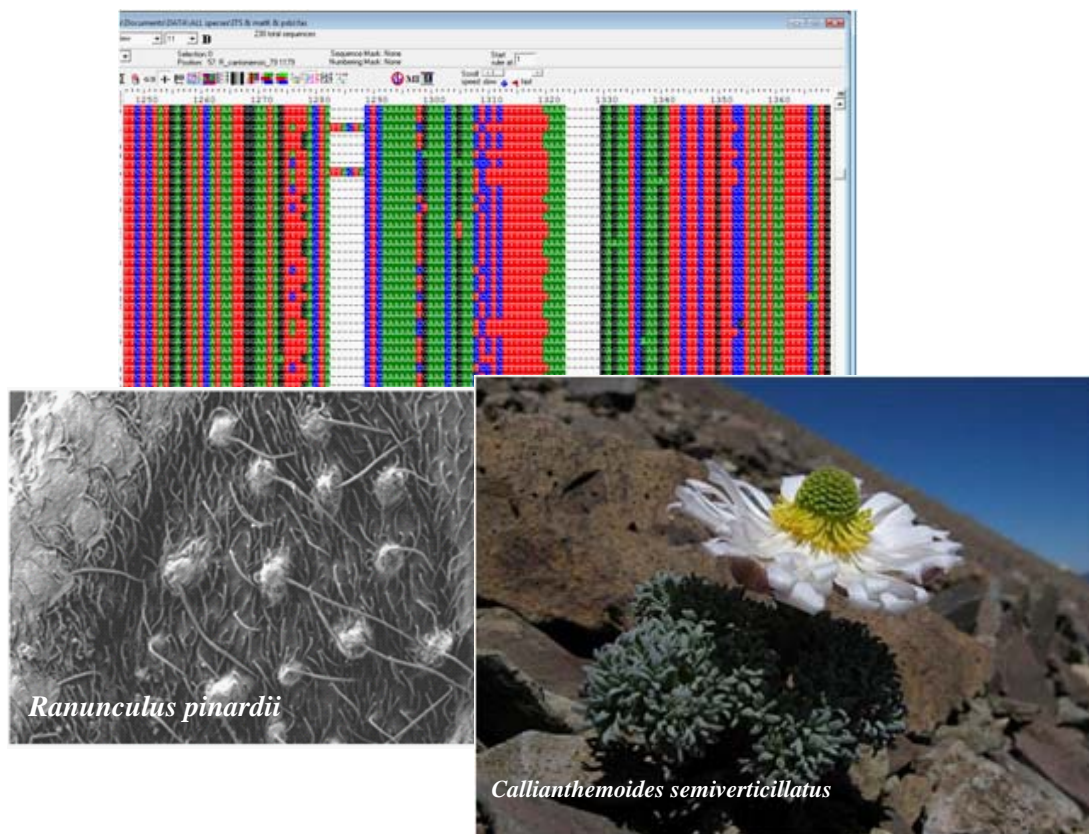
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>Arcteranthis cooleyae</i>	0	1	0	0	1	0	1	0	0	1	—	2	0	1	0	1	0
<i>Beckwithia andersonii</i>	1	0	2	0	0	0	1	0	0	1	1	4	0	1	1	0	0
<i>Callianthemoides semiverticillatus</i>	1	1	0	0	0	0	1	0	0	2	0	4	0	1	1	0	0
<i>Ceratocephala falcata</i>	0	0	2	0	0	0	1	0	0	0&1	0	1	0	1	0	1&2	0
<i>Ceratocephala orthoceras</i>	0	0	2	0	0	0	1	0	0	0&1	0	1	0	1	0	1&2	0
<i>Coptidium lapponicum</i>	1	0	1	0	0	0	0	0	0	2	0	2	0	1	0	0	0
<i>Coptidium pallasii</i>	1	0	1	0	0	0	0	0	0	2	1	2	0	1	0	0	0
<i>Cyrtorhyncha ranunculina</i>	1	1	0	0	0	0	1	0	0	2	0	0	0	1	0	0	0
<i>Ficaria fascicularis</i>	1	1	0	0	0	0	0	0	0	2	0	1	0	1	0	0	0
<i>Ficaria verna</i>	1	1	0	0	0	0	0	0	0	2	0	1	0	1	1	0	0
<i>Halerpestes cymbalaria</i>	1	0&1	0&2	0	0	0	2	0	0	1&2	0	2	0	1	1	1	0
<i>H. uniflora</i>	1	0	2	0	0	0	0	0	0	2	0	2	0	1	1	1	0
<i>Hamadryas delfinii</i>	1	0	2	1	0	0	2	0	0	2	0	2	0	1	0	1	0
<i>Krapfia clypeata</i>	1	1	0	0	0	<b>1</b>	2	0	0	2	1	2	<b>0&amp;1</b>	<b>0</b>	0	0	0
<i>Kumlienia hystricula</i>	1	0	2	0	1	0	1	0	0	2	—	2	0	1	0	1	0
<i>Laccopetalum giganteum</i>	1	0	2	0	0	<b>1</b>	1	0	0	1	1	2	<b>1</b>	<b>0</b>	0	0	0
<i>Myosurus minimus</i>	0	0	2	0	0	0	1	<b>1</b>	0	1	0	2	0	1	0	<b>3</b>	<b>1</b>
<i>Oxygraphis polypetala</i>	1	0	2	0	0	0	1	0	0	2	0	0	0	1	0	1	0
<i>Peltocalathos baurii</i>	1	1	0	0	0	0	1	0	0	2	0	2	0	1	1	0	0
<i>Ranunculus acris</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0
<i>R. apiifolius</i>	0	1	0	0	0	0	1	0	0	1	1	2	0	1	0	1	0
<i>R. arvensis</i>	0	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0
<i>R. asiaticus</i>	1	0&1	0	0	0	0	1	0	0	1	0	0&1	0	1	0	2	0
<i>R. bonariensis</i>	1	0	1	0	0	0	0	0	0	0	0	2	0	1	1	0	0
<i>R. brevifolius</i>	1	1	0	0	0	0	1	0	0	1	0	2	0	1	0	0	0
<i>R. breyninus</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0
<i>R. camissonis</i>	1	1	0	0	0	0	1	0	0	1	1	5	0	1	0	0	0
<i>R. caucasicus</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0
<i>R. cheirophyllus</i>	0	1	1	0	0	0	0	0	0	0	0	2	0	1	0	0	0
<i>R. flammula</i>	1	1	0	0	0	0	1	0	0	1	0	2	0	1	0	0	0
<i>R. formosomontanus</i>	1	1	0	0	0	0	1	0	0	1&2	0	0	0	1	0	0	0
<i>R. glacialis</i>	1	1	0	0	0	0	1	0	0	1	1	5	0	1	0	0	0
<i>R. kuepferi</i>	1	1	0	0	0	0	1	0	0	1	1	5	0	1	1	1	0
<i>R. lyallii</i>	1	1	0	0	0	0	1	0	0	2	1	4	0	1	1	0	0
<i>R. maclovianus</i>	1	0&1	0&2	0	0	0	1	0	0	1	0	1	0	1	0	0	0
<i>R. natans</i>	1	0	1	0	0	0	1	0	0	1	0	2&4	0	1	1	0	0
<i>R. nivalis</i>	1	0&1	0	0	0	0	1	0	0	1	0	2	0	1	0	1	0
<i>R. oxyspermus</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0
<i>R. papyrocarpus</i>	1	1	0	0	0	0	1	0	0	2	0	2	0	1	0	0	0
<i>R. parnassifolius</i>	1	1	0	0	0	0	1	0	0	1	1	5	0	1	0	0	0
<i>R. pedatifidus</i>	1	1	0	0	0	0	1	0	0	1	0	2	0	1	1	1	0
<i>R. peltatus</i>	0&1	0	1	0	0	0	1	0	0	1	0	3&4	0	1	1	0	0
<i>R. pensylvanicus</i>	0	1	0	0	0	0	1	0	0	1	0	1	0	1	1	1	0
<i>R. pinardii</i>	0	1	0	0	0	0	1	0	0	1	0	?	?	1	1	0	0
<i>R. polyanthemos</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	1	0	0
<i>R. pyrenaeus</i>	1	1	0	0	0	0	1	0	0	1	1	5	0	1	0	0	0
<i>R. rufosepalus</i>	1	0&1	0	0	0	0	1	0	0	1	0	2	0	1	0&1	0	0
<i>R. sceleratus</i>	0	1	0	0	0	0	1	0	0	1&2	0	4	0	1	0	1	0
<i>R. sphaerospermus</i>	0	0	1	0	0	0	1	0	0	1	1	4&5	0	1	1	0	0
<i>R. thora</i>	1	1	0	0	0	0	1	0	0	1	0	2	0	1	0	0	0
<i>R. trichophyllus</i>	0&1	0	1	0	0	0	1	0	0	1	1	3&4	0	1	1	0	0
<i>R. uncinatus</i>	1	1	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0
<i>Trautvetteria grandis</i>	1	1	0	0	1	0	0	0	1	—	—	—	—	1	1	0	0

Taxon	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
<i>Arcteranthus cooleyae</i>	1	1	0	1	0	1	0	0	0	0	1	0	1	0	0	2
<i>Beckwithia andersonii</i>	1	0	3	1	?	1	0&2	0	0	0	0	0	1	1	?	0&2&4
<i>Callianthemoides semiverticillatus</i>	1	1	2	1	2	1	0	0	0	0	0	0	2	2	0	4
<i>Ceratocephala falcata</i>	0	0	3	2	0	1	0	0	0	2	0	0	2	0	1	4
<i>Ceratocephala orthoceras</i>	0	0	3	2	0	1	0	0	0	2	0	0	2	0	1	2
<i>Coptidium lapponicum</i>	1	0	2	1	0	0	0	0	0	0	0	0	1	0	0	4
<i>Coptidium pallasii</i>	1	0	2	1	0	0	0	0	0	0	0	0	1	0	0	5
<i>Cyrtorhyncha ranunculina</i>	1	1	1	0	1	1	0&2	0	0	0	0	0	1	0	0	0&3
<i>Ficaria fascicularis</i>	1	0	2	1	0	1	0	0	0	0	0	1	3	3	0	0&2&4
<i>Ficaria verna</i>	1	0	2	1	0	1	0	0	0	2	0	1	3	3	0	2
<i>Halerpestes cymbalaria</i>	1	1	1	1	2	1	0	0	0	0	0	0	1	2	0	2
<i>H. uniflora</i>	1	1	2	1	?	1	0	0	0	0	0	0	1	2	0	2
<i>Hamadryas delfinii</i>	1	1	2	1	?	1	0	0	0	0	0	0	1	0	?	2
<i>Krapfia clypeata</i>	1	0	0	1	?	1	?	0	0	1	0	0	2	0	?	5
<i>Kumlienia hystricula</i>	1	1	2	1	1	1	0	0	0	1	0	0	1	0	?	4
<i>Laccopetalum giganteum</i>	1	0	0	1	0	1	?	0	0	0	0	0	0	0	?	5
<i>Myosurus minimus</i>	1	0	0	1	0	1	3	0	0	0	2	0	1	0	0	2
<i>Oxygraphis polypetala</i>	1	1	0	0	1	1	0	0	0	0	0	0	1	0	0	2
<i>Peltocalathos baurii</i>	1	1	2	1	1	1	0	0	0	0	1	0	1	0	0	?
<i>Ranunculus acris</i>	1	0	1	0	0	1	2	0	0	0	1	0	1	1	1	4
<i>R. apiifolius</i>	1	0	0	1	0	1	0	0	0	0	1	0	1	1	0	4
<i>R. arvensis</i>	1	0	3	0	0	1	1	1	0	0	1	0	1	0	0	5
<i>R. asiaticus</i>	1	0	1	0	0	1	2	0	0	0	1	0	1	2	0	5
<i>R. bonariensis</i>	1	0	0	1	0	1	2	0	0	0	0	0	1	1	?	?
<i>R. brevifolius</i>	1	3	3	1	0	1	0	0	0	0	0	0	1	0	0	?
<i>R. breynius</i>	1	0	2	0	0	1	2	0	0	0	1	0	1	0	0	?
<i>R. camissonis</i>	1	3	3	1	0	1	0	0	0	0	1	0	1	0	?	1
<i>R. caucasicus</i>	1	0	2	0	0	1	2	0	0	0	1	0	1	0	0	?
<i>R. cheirophyllus</i>	1	0	1	1	0	1	2	0	0	0	0	0	0	1	0	?
<i>R. flammula</i>	1	0	0	1	0	1	2	0	0	0	1	0	1	0	0	2
<i>R. formosomontanus</i>	1	0	1	1	0	1	2	0	0	0	0	0	1	1	0	?
<i>R. glacialis</i>	1	3	2	1	0	1	0	0	0	0	2	0	0	2	0	2
<i>R. kuepferi</i>	1	3	1	1	0	1	0	0	0	0	0	0	1	0	0	?
<i>R. lyallii</i>	1	0	2	1	0	1	0	0	0	2	1	0	2	0	0	2
<i>R. maclovianus</i>	1	0	1	1	0	1	2	0	0	1	0	0	1	0	?	?
<i>R. natans</i>	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	?
<i>R. nivalis</i>	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	?
<i>R. oxyspermus</i>	1	0	2	0	0	1	2	0	0	0	0	0	0	2	0	?
<i>R. papyrocarpus</i>	1	0	2	0	0	1	2	0	0	0	2	0	1	1	?	4
<i>R. parnassifolius</i>	1	3	2	1	0	1	0	0	0	0	0	0	1	0	0	?
<i>R. pedatifidus</i>	1	0	1	1	0	1	2	0	0	2	0	0	1	0	0	?
<i>R. peltatus</i>	1	0	1	1	0	1	0	0	1	0&2	1	0	1	0	0	?
<i>R. pensylvanicus</i>	1	0	1	0	0	1	2	0	0	0	1	0	1	0	0	0&4
<i>R. pinardii</i>	1	0	3	0	0	1	1	1	0	2	1	0	2	2	?	?
<i>R. polyanthemus</i>	1	0	1	0	0	1	2	0	0	0	1	0	1	0	0	2
<i>R. pyrenaicus</i>	1	0	1&2	1	0	1	0	0	0	0	0	0	1	0	0	2
<i>R. rufosepalus</i>	1	0	1	1	0	1	2	0	0	0	0	0	1	0	?	?
<i>R. sceleratus</i>	1	0	0	0	?	1	2	0	0&1	0	0	0	1	1	0	2
<i>R. sphaerospermus</i>	1	0	1	1	0	1	0	0	1	0	1	0	1	0	0	?
<i>R. thora</i>	1	3	2	1	0	1	0	0	0	0	0	0	1	0	0	2
<i>R. trichophyllus</i>	1	0	1	1	0	1	0	0	1	0&2	1	0	1	0	0	2
<i>R. uncinatus</i>	1	0	1	0	0	1	2	0	0	0	1	0	1	0	0	?
<i>Trautvetteria grandis</i>	1	1	1	3	2	1	0	0	0	1	2	0	1	0	0	0

## Chapter 3

### Northern Hemisphere origin, transoceanic dispersal, and diversification of Ranunculeae (Ranunculaceae) in the Tertiary\*

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## ABSTRACT

**Aim** The role of dispersal vs. vicariance for plant distribution patterns has long been disputed. We study temporal and spatial diversification of Ranunculeae, an almost cosmopolitan tribe comprising 19 genera, to understand the processes that have resulted in the present inter-continental disjunctions.

**Location** Our biogeographical study includes 18 genera and altogether 85 species from all continents (except Antarctica).

**Methods** Based on phylogenetic analyses of nuclear and chloroplast DNA sequences we develop a temporal-spatial framework for the reconstruction of the biogeographical history of Ranunculeae. To estimate divergence dates, Bayesian uncorrelated rates analyses and five calibration points are used. The age of split of *Ranunculus* and *Xanthorhiza* provides an external calibration point and the age of divergence of four species of *Ranunculus* were used as internal calibrations point. A parsimony-based dispersal-vicariance method (DIVA), a maximum likelihood-based method (Lagrange), and Mesquite are used for reconstructing ancestral areas. Six areas corresponding to continents were delimited.

**Results** The reconstruction of ancestral areas is congruent in the DIVA and Lagrange analyses in most nodes, only Mesquite reveals equivocal results at deep nodes. Our study suggests a Northern Hemisphere origin for the Ranunculeae in the Eocene and a weakly supported vicariance event between North America and Eurasia. The Eurasian clade diversified between the late Eocene and the late Miocene, with at least three independent migrations to the Southern hemisphere. The North American clade diversified in the Miocene and moved later to Eurasia, South America, and Africa.

**Main conclusions** Ranunculeae diversified between the late Eocene and the late Miocene. During this time period, the main oceanic barriers already existed between continents and dispersal is the most likely explanation for the current distribution of the tribe. In the Southern Hemisphere, a vicariance model following the breakup of the Gondwanaland is clearly rejected. Dispersals between continents must have occurred via migration over land bridges, or via long distance dispersal.

**Keywords** Ranunculeae, molecular dating, vicariance, long-distance dispersal, transoceanic dispersal.

## INTRODUCTION

Before 1960, most biogeographers believed that allopatric speciation was the main driver of diversification, e.g. ancestors of species dispersed across barriers, then became isolated, and evolved into new species (Udvardy, 1969). For centuries, dispersal was the dominant explanation for the distribution of organisms, but the recognition of plate tectonics led to vicariance being seen a more probable explanation than dispersal (Wiley, 1998; de Queiroz, 2005). Today it is widely accepted that disjunct distributions can be explained either by fragmentation of widespread ancestors by vicariant (isolating) events or by dispersal across a barrier. Molecular-based phylogenetic studies based on DNA sequences and estimates of divergence times of lineages supported the role of dispersal as a primary process shaping distribution patterns in both animals and plants (reviewed by de Queiroz, 2005). These studies provide a huge amount of evidence supporting a hypothesis of transoceanic dispersal versus vicariance (Givnish & Renner, 2004; Sanmartin & Ronquist, 2004; de Queiroz, 2005).

Widespread and species-rich plant families like the Ranunculaceae provide model system for studying biogeographical processes. This family has been considered as one of the most basal families within the eudicots (Simpson, 2006; Heywood & al., 2007), with a crown age of c. 75 my (Anderson & al., 2005). There are different opinions about the origin of the Ranunculaceae: the Neogene warm-temperate flora (Popov, 1927), the early Tertiary tropical flora (Scharfetter, 1953). The paleobotanical record reflects the considerable differentiation of Ranunculaceae and their radiation throughout the world during the Neogene in the Northern Hemisphere (Ziman & Keener, 1989). Although Ziman and Keener (1989) proposed the origin of some tribes within the ancient floras of eastern Asia (e.g. Anemoneae, Clematideae), or in North America (e.g. Hydrastideae), they emphasized that it is difficult to pinpoint the origin of some tribes such as Ranunculeae.

Ranunculeae DC. comprise 19 genera (K. Emadzade *et al.*, in press) around 650 species (Tamura, 1995). Most species of this cosmopolitan tribe are adapted to temperate and cold climates and occur in mountain regions of the world. A number of molecular phylogenetic studies within Ranunculaceae suggest that this tribe is monophyletic (Hoot, 1995, Hoot *et al.*, 2008; Johansson, 1995, 1998; Ro *et al.*, 1997; Lehnebach *et al.*, 2006; Wang *et al.*, 2009). Former molecular phylogenetic studies on Ranunculeae have concentrated either on certain geographical areas (e.g. New Zealand, Lockhart *et al.*, 2001; Mediterranean area, Paun *et al.*, 2005; Southern Hemisphere, Lehnebach, 2008; Africa, Gehrke & Linder, 2009; Arctic, Hoffmann *et al.*, 2010) or certain genera of the tribe (e.g. *Ranunculus*, Hörandl *et al.*, 2005, *Laccopetalum*, Lehnebach *et al.*, 2006; *Hamadryas*, Hoot *et al.*, 2008). Phylogenetic

relationships and taxonomy of the tribe have been established based on molecular and morphological data (K. Emadzade *et al.*, in press). A complete biogeographical study of all genera of the tribe is still missing.

The distribution patterns in this tribe provide a model system for studying vicariance vs. dispersal. *Ranunculus* is the only genus distributed in all continents (Fig. 1h). Most other genera have very restricted distributions, and many of the monotypic genera are endemic to small areas, such as *Cyrtothyncha* and *Kumlien* (North America; Fig. 1c, e), *Laccopetalum* (South America; Fig. 1f), and *Peltocalathos* (South Africa; Fig. 1g). Some genera, such as *Ceratocephala* and *Myosurus*, are mainly distributed in the Northern Hemisphere (Fig. 1b, f), but some species occur far away from the main area in the Southern Hemisphere (Tamura, 1995). *Trautvetteria* has a disjunct distribution in eastern Asia and eastern and western North America (Fig. 1h). The species of this genus have been considered as relics of the Tertiary temperate flora (Thorne, 1973). It is interesting that some of the closely related taxa in Ranunculeae occur on different continents, e.g. *Callianthemoides*, *Hamadryas* and *Peltocalathos* (Fig. 1b, e).

The origin of Ranunculeae probably dates back to the mid Eocene (Paun *et al.*, 2005; Hoffmann *et al.*, 2010). This period was important in the evolution of all biota due to great tectonic movements and climatic fluctuations. In this time, the split up of Gondwanaland had already been completed, but North America and Eurasia still had connections via Greenland. The climate had cooled down and extensive glaciations had occurred by the end of the period (Tiffney, 2000; McLoughlin, 2001; Milne & Abbott, 2002). However, previous age estimates for the tribe suffered from incomplete sampling of genera and the lack of internal calibration points. Therefore, the timing of biogeographical events has remained tentative.

The combination of phylogenetic data with spatial-temporal data provides a strong basis for understanding the biogeographical history of the group (Donoghue & Moore, 2003; Renner, 2005; Kelly *et al.*, 2009). We combine here the results from molecular dating and biogeographical analyses to provide a comprehensive hypothesis of the history of Ranunculeae. The aims of this study are to 1) reconstruct divergence dates within Ranunculeae; 2) to localize the center of origin for the tribe; 3) point out the main migration routes, and 4) to reconstruct the main factor(s) shaping the modern distribution of the tribe, including the relative role of long-distance dispersal and vicariance.

## MATERIAL AND METHODS

### Taxon sampling

A total of 85 species of Ranunculeae was included in the analyses, representing 18 of the 19 genera of the tribe (K. Emadzade *et al.*, in press), as well as three outgroup taxa (Appendix S1). Only the monotypic genus *Paroxygraphis*, endemic to the Himalayas, was not included because material was not available. 143 new sequences of a nuclear marker (ITS of the nuclear ribosomal DNA), chloroplast markers (*matK/trnK*, and *psbJ-petA*) were obtained from new samples and combined with data from previous studies (Hörandl *et al.*, 2005; Paun *et al.*, 2005; Gehrke & Linder, 2009; Hoffmann *et al.*, 2010). Voucher information and GenBank accession numbers are provided in Appendix S1. We included many species of *Ranunculus* for age estimates because four internal calibration points were available in this genus (Fig. 2, arrows). Biogeographical analyses of *Ranunculus* will be presented elsewhere.

### DNA extraction, amplification, sequencing, and phylogenetic analysis

Total genomic DNA from silica-dried or herbarium material was extracted using a modified CTAB technique (Doyle & Doyle, 1987). For amplification and sequencing of the nr ITS, *matK/trnK*, and *psbJ-petA* regions the protocol of Hörandl *et al.* (2005), Paun *et al.* (2005), and Shaw *et al.* (2007) were used, respectively. Sequence alignment and molecular phylogenetic reconstruction were performed as described in K. Emadzade *et al.* (in press).

### Molecular age estimation

We used the Bayesian relaxed clock methodology to calibrate a temporal framework of the phylogeny (Drummond & Rambaut, 2007). The lack of pre-quaternary species-specific fossils in Ranunculeae makes age calibration difficult. Records of fossil pollen and leaves of *Ranunculus* in different areas (Martin-Closas, 2003; Kalis *et al.*, 2006) cannot be reliably assigned to certain species because of the great intra- and interspecific variation of these characters (E. Hörandl & K. Emadzade, in prep.). Uncertainty of fossil calibration is a source of error in the dating (Gandolfo *et al.*, 2008).

We used *matK* as a maternally inherited gene rather than ITS or combined sequences for age estimates to avoid the problems of recombination and concerted evolution in the nuclear marker. Moreover, *matK* is more conserved over the evolutionary divergences studied and the only marker available for dating the split of *Xanthorrhiza* and *Ranunculus*. Because of a high

percentage of missing data in the selected region, we excluded *Trautvetteria* and *Arcteranthus* from the data set.

We calibrated five nodes of the tree using external information to calculate age estimates of Ranunculeae. The age of the split of *Ranunculus* (Ranunculeae) and *Xanthorhiza* (Dichocarpeae) was estimated between 51 to 66 My (Wikström *et al.*, 2001). The ages of two oceanic islands are assumed to be maximum ages for the split of island endemics from their closest relatives, i.e. *Ranunculus hawaiiensis* endemic to the Big Island of the Hawaiian archipelago, ca 0.5 My (Price & Clague, 2002) and *R. caprarum* endemic to Masafuera (one of the three islands of the Juan Fernández archipelago), 1-2 My (Stuessy *et al.*, 1984). We further used the divergence time between *Ranunculus cassubicifolius* and *R. carpaticola* (317,000 yr; Hörandl, 2004) and *R. carpaticola* and *R. notabilis* (914,000 yr; Hörandl, 2004), both based on isoenzyme studies. Since we use these calibration points for the dating of ectopic events, we avoid circular reasoning for biogeographical hypotheses.

Divergence times were calculated using a relaxed clock model (Drummond *et al.*, 2006) as implemented in the computer program BEAST v1.4.5 (Drummond & Rambaut, 2007). The partitioned BEAST.xml input file was created with BEAUti v1.4.5 (Drummond & Rambaut, 2007). The *matK* dataset was tested using MrModeltest 2.2 (Nylander, 2004) to determine the sequence evolution model that best described the present data. A GTR+I+ $\Gamma$  substitution model and the gamma distribution were modeled with four categories. A Yule prior on branching rates was employed and four independent MCMC analyses were each run for 100,000,000 generations, sampling every 1000 generations. Convergence and acceptable mixing of the sampled parameters was checked using the program Tracer 1.2 (Rambaut & Drummond, 2003). After discarding the burn-in steps, the four runs were combined using TreeAnnotator (Rambaut & Drummond, 2002) to obtain an estimate of the posterior probability distribution of the divergence dates of the ancestral nodes.

### **Optimization of ancestral distributions**

We used three methods: a parsimony-based method (DIVA vs. 1.1, Ronquist, 1997); a maximum likelihood-based method (Lagrange v. 2.0.1, Ree & Smith, 2008), and Mesquite vs. 2.6, Maddison & Maddison, 2009), to infer vicariance and dispersal events.

Dispersal-Vicariance Analysis optimizes distributions for each node of the tree by minimizing the number of assumed dispersals and extinctions and favoring the vicariance events (Ronquist, 1996, 1997). This program reconstructs widespread ancestral distributions instead restricting these distributions to single areas. Moreover, because allopatric speciation

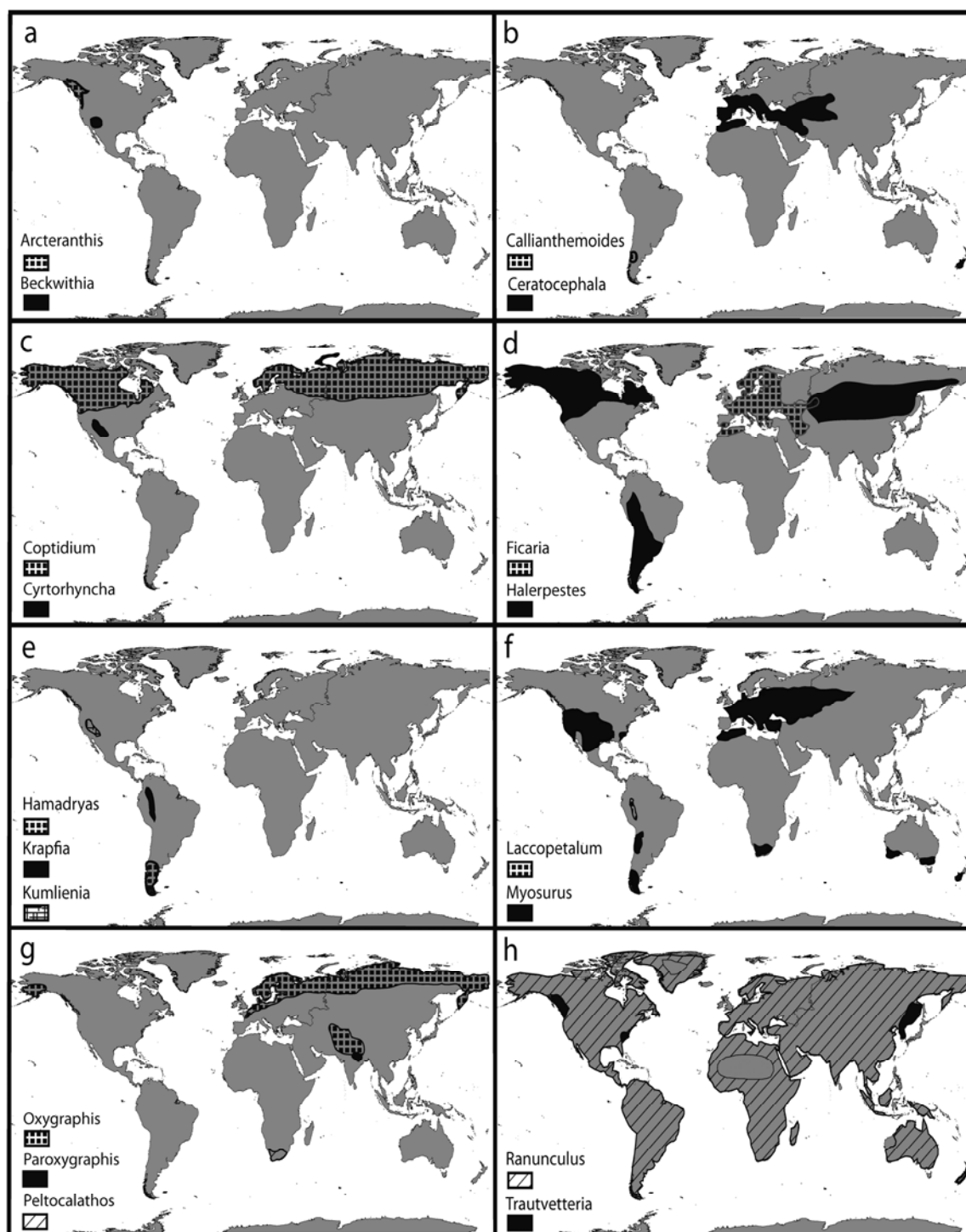
by vicariance is the null model in DIVA, vicariance and range division would always be the preferred explanation if ancestors are widespread (Sanmartin, 2006). Because of the presence of widespread taxa (e.g. *Ranunculus* and *Halerpestes*) additional to unconstrained areas, a limit of three areas was set (maxareas=3) in DIVA.

A newly developed method represents a significant advance in biogeographic methodology by using a maximum likelihood (ML) statistical model (Lagrange; Ree & Smith, 2008). This method includes information from biological and biotic factors by calculating the likelihood of biogeographic routes and areas occupied by most common ancestor for a given phylogenetic tree topology and the present distributions of taxa. For example, the rate of dispersal and local extinction, the time of lineage surviving, and the probabilities of dispersal between geographic ranges at different geological times (Ree *et al.*, 2005) can all be used in the reconstruction. We further reconstruct ancestral states based on parsimony using Mesquite (Maddison & Maddison, 2009). Based on all the combined sequence data, we produced a phylogenetic tree as described in K. Emadzade *et al.* (in press) with PAUP\* version 4.0b8 (Swofford, 2002) for DIVA and Mesquite, and an ultrametric tree using the Bayesian analysis program BEAST v1.4.5 (Drummond & Rambaut, 2007) for Lagrange. Then we reduced the number of species of each genus to a single in Mesquite (Maddison & Maddison, 2009).

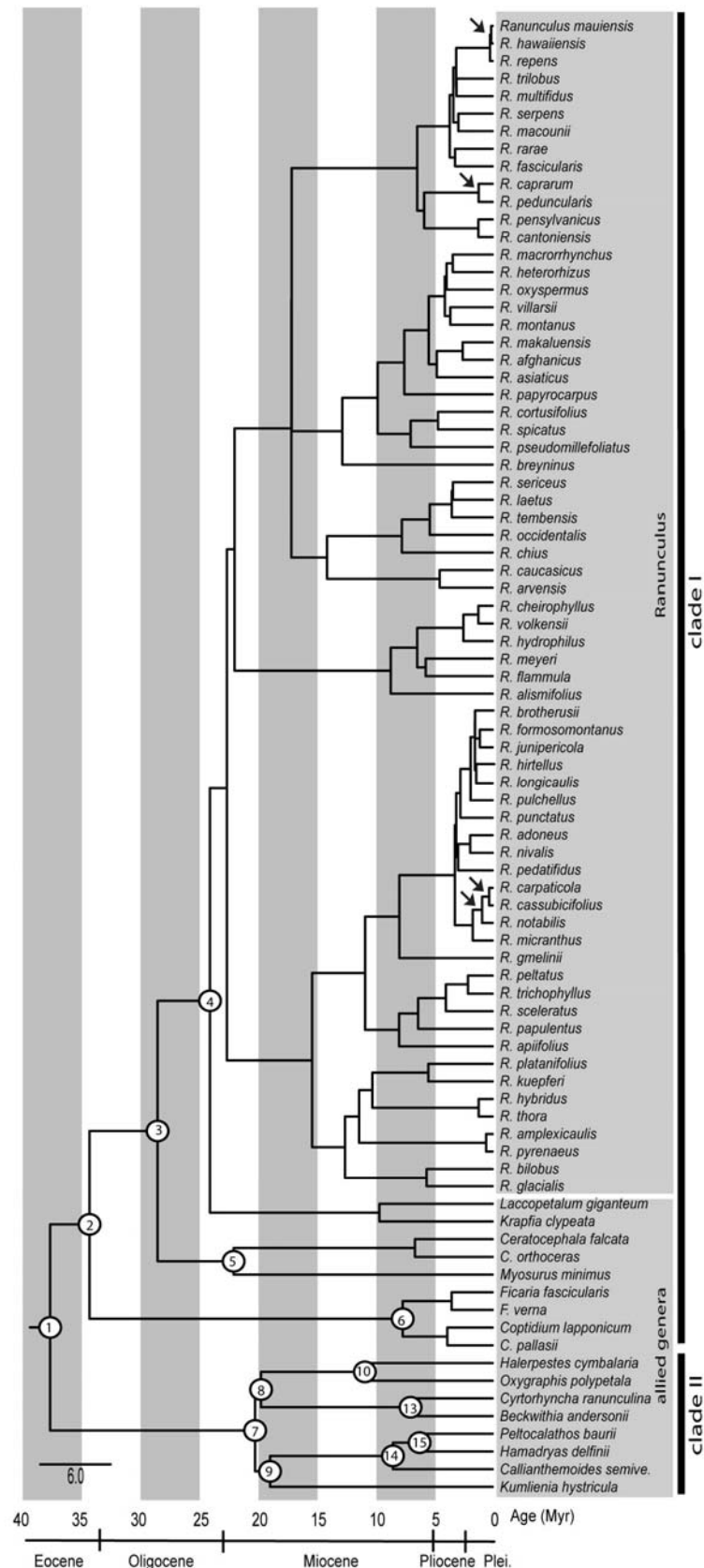
Distribution data were compiled from the literature (e.g., Ovczinnikov, 1937; Meusel *et al.*, 1965; Lourteig, 1984; Iranshahr *et al.*, 1992; Tamura, 1995; Whittemore, 1997; Wencai & Gilbert, 2001). Areas were delimited by continental divisions as: Africa (AF), Asia (ASI), Europe (EUR), N. America (NA), S. America (SA), and Oceania (OCE). The distribution of each genus of Ranunculeae included in this analysis is shown in Figs. 1 and 3.

To illustrate possible historical scenarios, migration ways and biogeography of Ranunculeae, maps were designed to show the respective position of continental plates at different time periods using a program provided by the Ocean Drilling Stratigraphic Network (ODSN; established by GEOMAR, Research Center for Marine Geosciences/ Kiel, and the Geological Institute of the University Bremen; see <http://www.odsn.de>) that is based on data used by Hay *et al.* (1999).





**Figure. 1.** Extant distribution of Ranunculeae. Genera are ordered alphabetically.



**Figure 2.** Chronogram of Ranunculeae using the *matK* dataset based on BEAST analyses. Numbers in circles referred to nodes of the tree in Figure 3. Nodes labeled refer to the positions of the four internal calibration points used (external calibration point and out groups are not shown). Geological time scale (Gradstein *et al.* 2004) is shown at the bottom.

## RESULTS

### Divergence time estimation

The *matK* chronogram (Fig. 2) and the highest posterior density intervals (HPD; Table 1) reveal the crown group age of the tribe and a split between two main clades (clade I and II) in the late Eocene (38.10 Myr; node 1). Clade I diversified between the late Eocene and the late Miocene (nodes 2-6). *Ranunculus* diverged from its South American sisters *Krapfia* and *Laccopetalum* already in the Late Oligocene (node 4). *Ceratocephala* split from *Myosurus* in the early Miocene (node 5), while *Ficaria* separated from *Coptidium* in the late Miocene (node 6).

In Clade II, the Eurasian genera diverged from North American sisters already in the early Miocene (node 8). *Kumlienia*, from western North America, apparently diverged from its Southern Hemisphere relatives (node 9) also in the early Miocene. The African genus *Peltocalathos* split from the Southern American *Hamadryas* in the late Miocene (node 15).

### Biogeographical data

Parsimony analysis of the combined data (ITS, *matK/trnK*, and *psbJ-petA*) set used for DIVA and Mesquite (Appendix S2) shows congruence with the tree produced with BEAST which was used for Lagrange. DIVA and Lagrange analyses overall revealed similar results (Table 1). These analyses suggest that Ranunculeae most likely originated in the Northern Hemisphere, and then split into two clades by vicariance (Fig. 3, clades I, II). However, this node has bootstrap a support less than 50. DIVA showed that the most recent common ancestor (MRCA) of clade I occupied Europe or Asia, and Lagrange confirmed that the MRCA of clade I occurred in Eurasia. Mesquite, however, reveals the origin of this node equivocal (Fig. 3, node 2). All three analyses show that the MRCA of clade II occurred in N. America (Fig. 3, node 7). Biogeographical analyses and present distribution of genera reveal that dispersal between continents can have occurred independently via different routes in different time periods such as: Eurasia to South America (Fig. 3, node 2 → 3), North America to South America (Fig. 3, node 9 → 14), and South America to Africa (Fig. 3, node 14 → 15).

DIVA analysis reconstructed Asia or Europe as ancestral area of the *Coptidium-Ficaria* clade (Eurasian distribution) and the *Ceratocephala-Myosurus* clade (mainly Northern Hemispheric distribution with some species in the Southern Hemisphere); however, Lagrange revealed that the MRCA of these clades occurred only in Asia (Fig. 3, nodes 5, 6). The node separating *Kumlienia* from other genera of clade II-b, and the node separating *Beckwithia* +

*Cyrtorhyncha* from other genera of clade II-a are all reconstructed with ancestral distributions in North America under all three geographical analyses.

Results of unconstrained and constrained analyses of DIVA show congruence except for node 4 (Table 1). Unconstrained analysis of DIVA placed the MRCA of node 4 in one of the 25 area combinations, but constrained area analysis (maxareas=3) reduced the number of combinations to 10 (Table 1, node 4). Optimal reconstruction required 24 dispersal events for the unconstrained analysis and 27 dispersals when maxareas was limited to 3.

## DISCUSSION

### Spatial-temporal diversification of the genera

Our age estimates refined the results of previous studies (Paun *et al.*, 2005; Hoffmann *et al.*, 2010). Mostly the ages of the nodes in our analysis fall between the ages of the two previous studies. The main difference between these studies is the crown group age of the tribe (40 My in Paun *et al.*, 2005; more than 50 My in Hoffmann *et al.*, 2010, and 38.10 My in the present study), which is probably due to different calibrations (Anderson *et al.*, 2005). Paun *et al.* (2005) used only the age of the split between *Ranunculus* and *Xanthorhiza* and Hoffmann *et al.* (2010) derived two calibration points from this study. Both studies used no internal calibration points, and applied different methods and data sets.

Our results suggest that the tribe most likely originated in the Northern Hemisphere, which has been inferred for other genera of Ranunculaceae as well (Schuettpeitz *et al.*, 2002; Schuettpeitz & Hoot, 2004). The crown group age and the split into the main two clades (Fig. 3, node 1) probably dates back to the late Eocene (38.10 Ma) which almost coincides with the break-up of the connection between Greenland and Europe (Tiffney, 2000). At the end of the Eocene all continents were close to present positions but still no connection existed between South America and North America (Sanmartin & Ronquist, 2004). Ranunculeae originated probably before the complete separation of Greenland and Europe and then diversified separately on both sides of the Atlantic (Fig. 4a).

The biogeographical analyses suggest multiple dispersal events from the Northern Hemisphere (in clade I from Eurasia, and in clade II from North America) to the Southern Hemisphere in the late Paleogene and the early Neogene. One of the migration routes from Eurasia to S. Hemisphere (S. America) happened probably in clade I (node 2 to 3 and/or 3 to 4, Fig. 3), in the Oligocene. The most parsimonious way is long distance dispersal (LDD) from Europe to S. America (Fig. 4b, arrow 2→3). Long distance dispersal over the Atlantic

Ocean has been suggested in other families with similar distributions as well ( e.g. Wendel & Albert, 1992; Coleman *et al.*, 2001, 2003; Tremetsberger *et al.*, 2005).

Another, less plausible scenario is a migration of the ancestor from Eurasia to S. America via N. America through the Bering Land Bridge (BLB) or the North Atlantic Land Bridge (NALB) or across the Atlantic by LDD. Then the colonization to South America could have been possible because the positions of North and South America have not changed so much since the Oligocene (Scotese, 2001). Later the ancestors may have gone extinct in North America.

Migrations from Eurasia to the Southern Hemisphere (South America, New Zealand and Australia) also happened in the *Myosurus-Ceratocephala* clade two times separately (Fig. 3, node 5). We cannot infer from our data whether already the ancestors of these genera arrived in the Southern hemisphere or whether this migration happened within genera. Nevertheless, according to the age of node 5 (late Oligocene/early Miocene), the occurrence of some species of *Myosurus* and *Ceratocephala* endemic to New Zealand (Garnock-Jones, 1984; Fig. 1b, f) must be explained by LDD or by migration via New Guinea and Australia (Fig. 4c, arrow 5). This route has been suggested not only for Australian (Armstrong, 2003) and New Zealand (Lehnebach, 2008) *Ranunculi*, but also for other taxa (Wanntorp & Wanntorp, 2003; Kadereit *et al.*, 2005). *Myosurus* could have migrated via LDD from Australia and New Zealand to South Africa and South America or the other way around (Fig. 4d). Alternatively, *Myosurus* could have moved from Eurasia to North America and then to the Southern hemisphere. The long time period since late Oligocene/early Miocene implies many possibilities for different migrations. In these genera, anthropochorous dispersal is also likely. However, further studies including more taxa and biogeographical analyses within these two genera are necessary to pinpoint the exact migration routes and biogeographic scenario within this clade.

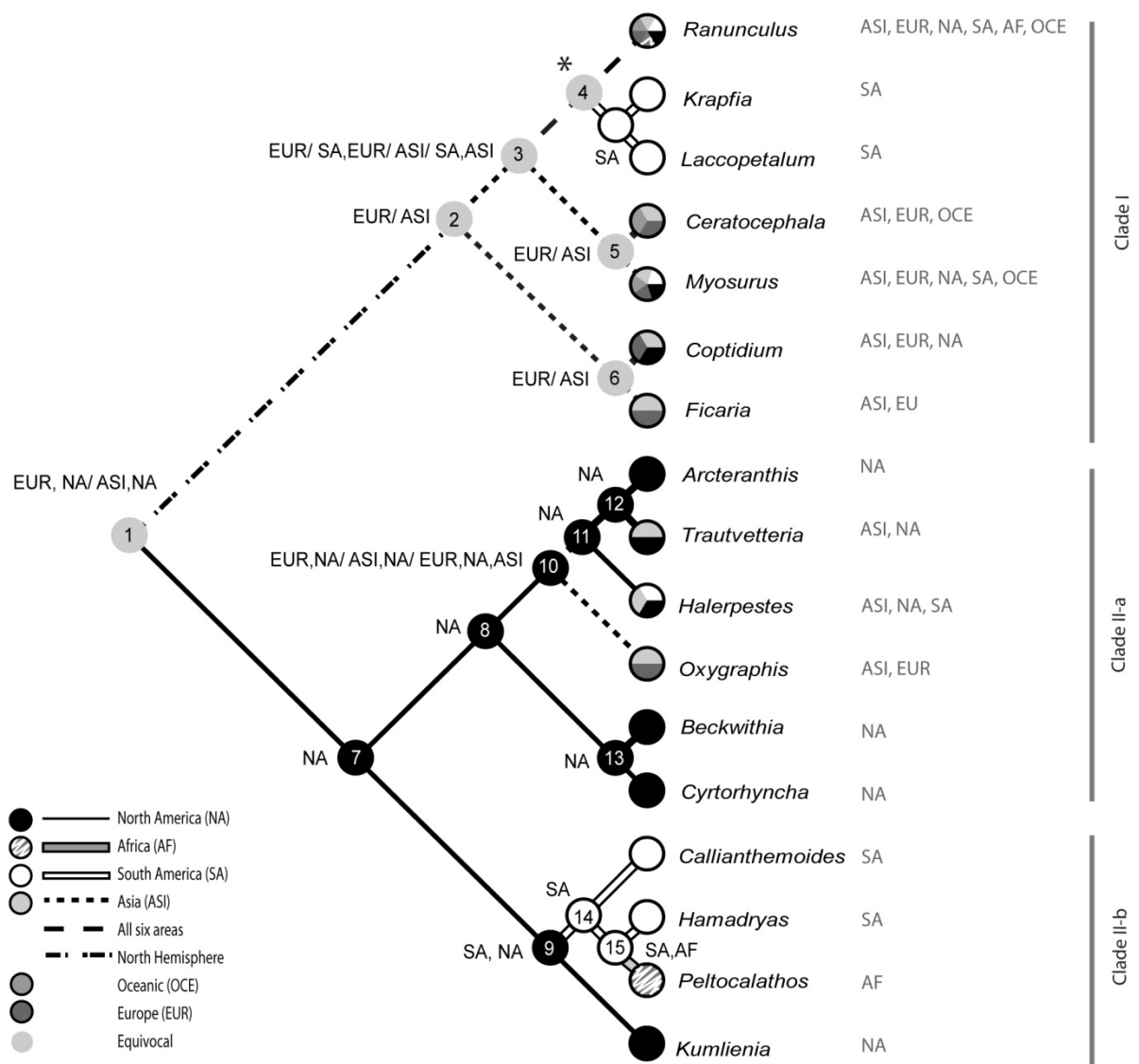
The MRCA of the *Coptidium-Ficaria* clade occurred in Eurasia, respectively, during the Miocene (Fig. 3, node 6). The presence of the descendants of this clade in the high Arctic (Fig. 1c), such as *Coptidium pallasii*, could be the result of recent migrations after the Pleistocene glaciations (Fig. 4c, arrows 6) or survival of the descendants in refugia during the glaciations, e.g. in the Bering Land Bridge (Hulten, 1937; Abbott & Brochmann, 2003). The Arctic was colonized multiple times by species of *Ranunculus* s.str. (Hoffmann *et al.*, 2010).

Due to the wide distribution of *Ranunculus* (Fig. 1h), DIVA analysis (in constrained and unconstrained analyses) revealed several possibilities for the place of occurrence of the most common ancestors of the *Ranunculus-Krapfia-Laccopetalum* clade (Fig. 3, node 4). Biogeography and age estimates of this large clade will be discussed in a separate paper.

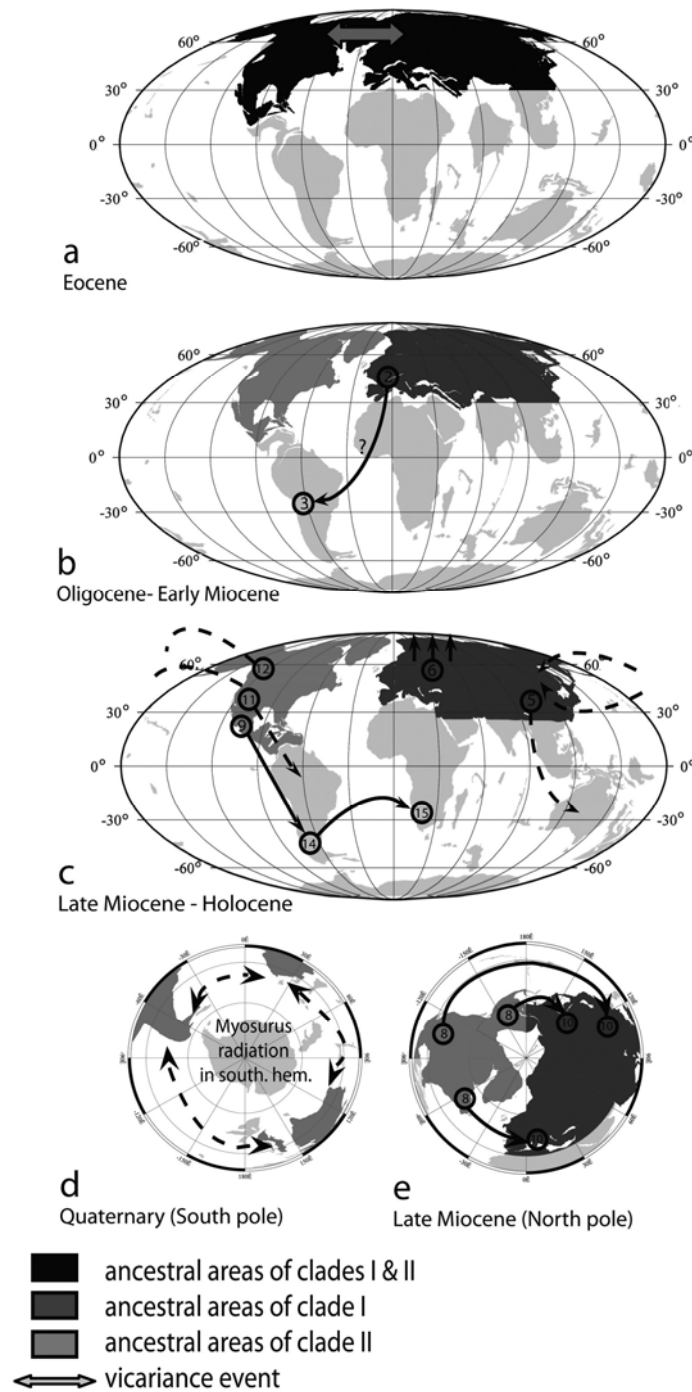
Patterns of distribution and history of migration routes in clade II are less complicated than in clade I. The MRCA of this clade occurred in North America in the early Miocene. Based on the extant distribution of *Halerpestes*, *Oxygraphis*, and *Trautvetteria* (Fig. 1d, g, h), dispersal events from N. America to Eurasia are likely. Most of the geographical evidence indicates that the North Atlantic Land Bridge (NALB) persisted till about 40 Ma and broke during the Eocene (Milne & Abbott, 2002). Nevertheless, the similarity between the flora of N. America and Europe and previous molecular studies suggest that some exchange of taxa could have continued until the Miocene (Wen, 1999; Hably *et al.*, 2000; Manos & Donoghue, 2001). On the other hand, the sea level was at least 100-150 m lower than present (Hallam, 1992), which may have allowed an exchange of taxa between the Old and the New World. We can assume that the plants could have been able to cross the NALB even later because they could have migrated using island chains as a bridge (Tiffney, 2000; Manos & Donoghue, 2001). Biological and geological studies indicate that the Bering Land Bridge (BLB) was open from the early Paleocene and closed at the late Miocene (Tiffney & Manchester, 2001). If we assume that this clade is probably too young (ca. 15 My, Fig. 2) for a migration from N. America to Eurasia via the NALB, migration via the BLB or LDD across the Atlantic or Pacific is more likely to have occurred (Fig. 4e, arrows 8→10). According to the area optimization of node 14 (Fig. 3) in South America in all analyses, migration from North to South America is well supported. During the Miocene, South America had considerable contact via the Panama Isthmian region with North America (Briggs, 1987). So the ancestor of clade II-b could easily migrate over this land bridge (Fig. 4c, arrow 9→14).

A close floristic relationship between Pacific North America and East Asia has been observed in many genera (Xiang *et al.*, 1998; Milne & Abbott, 2002). *Trautvetteria* is one example of this transoceanic connection. Our data suggest migration of the most common ancestor of *Trautvetteria* from North America to Asia (Fig. 4c, arrow 12), which confirms Gray's (1878) hypothesis. He suggested that a continuous flora existed across the BLB and the occurrence of the same taxa in different continents could be explained by the break-up of this flora during the Pleistocene glaciations. The same migration route was probably used by *Halerpestes* as well (Fig. 4c, arrow 11).

The closely related genera *Hamadryas* and *Peltocalathos*, endemic to S. America and S. Africa, respectively, are probably ca. 6.23 Ma old (Fig. 2). According to this age estimate, LDD from South America to South Africa is more likely than a vicariance model due to the Gondwanaland breakup which has happened 130-100 Ma (Lomolino *et al.*, 2006).



**Figure 3.** Biogeographical optimization performed with the software DIVA, Lagrange, and Mesquite of Ranunculeae. This tree is based on the ITS, *matK/trnK*, and *psbJ-petA* dataset. Relevant nodes are numbered (in circles). The distribution of genera, as coded for biogeographical analyses, is indicated next to each taxon. Most recent common ancestors reconstructed by DIVA are indicated on each node. Different lines show the migration routes suggested by Lagrange. Shading shows ancestral area reconstruction under parsimony in Mesquite. Coded as stated in the figure: **NA**, N. America; **SA**, S. America; **EUR**, Europe; **ASI**, Asia; **AF**, S. Africa; **OCE**, New Zealand, Australia. Asterisk, several combinations of areas have been optimized by DIVA which is presented in Table 1, node 4.



**Figure 4.** Historical biogeography of Ranunculeae. The maps show the position of plates in different geological periods and ancestral areas inferred from Figure 3. Solid arrows depict predominant dispersal events. Numbers in circles referred to nodes of the tree in Figure 3. Dashed arrows indicate hypothetical recent dispersal events within genera.

### **Long distance dispersal or vicariance**

The biogeographical scenarios presented here mainly suggest migrations over land bridges and transoceanic dispersal rather than vicariance events in the tribe. In the other hand, it presents another example of Northern hemisphere origin of temperate plants followed by the expansion towards the Southern Hemisphere (Bell & Donoghue, 2005; Inda *et al.*, 2008). Northern Hemispheric origin and dispersal to the Southern Hemisphere is supported by similar links found within other genera of Ranunculaceae (*Anemone*; Schuettpehlz *et al.*, 2002; *Caltha*, Schuettpehlz & Hoot, 2004). The breakup of the Gondwanaland has been assumed to be the main factor of the Southern Hemisphere distribution of these genera (Schuettpehlz *et al.*, 2002; Schuettpehlz & Hoot, 2004). Recent molecular and phylogeny studies on plants rejected the breakup of Gondwanaland as the main factor of modern distribution of some taxa and showed that dispersal may be more effective than previously recognized (de Queiroz, 2005; Levin, 2006; Heaney, 2007; Harbaugh *et al.*, 2009; Schaefer *et al.*, 2009). De Queiroz (2005) demonstrated the importance of transoceanic dispersal for the distribution of extant taxa.

Recent analyses revealed that the historical biogeography of the Northern (Wen, 1999, 2001; Donoghue *et al.*, 2001; Xiang & Soltis, 2001) and Southern Hemisphere (Sanmartín & Ronquist, 2004) cannot be explained by a simple vicariance model and dispersal was a main factor for biogeographical patterns in the Northern Hemisphere. Knapp *et al.* (2005) rejected the hypothesis that present-day distribution patterns of *Nothofagus* can be explained by continental drift following the breakup of Gondwana. He emphasized that LDD is more likely to describe the modern distribution patterns in the Southern Hemisphere.

Long distance dispersal of plant via seeds or propagules to isolated islands, followed by speciation, is a main factor in richness of their flora (Wagner & Funk, 1995; Cowie & Holland, 2006; Lomolino *et al.*, 2006). Island endemism could be another evidence for the ability of plant migration. The presence of endemic species of *Ranunculus* in some oceanic islands, far away from the continents (e.g. Hawaii Islands, Juan Fernandez Islands, and Canarian Islands), also confirm that LDD is possible in this tribe. Smith (1986) showed that only one successful long-distance dispersal and establishment event needs to occur approximately every 10,000 years to explain the species richness observed in the Australasian alpine and tropic-alpine flora. LDD does neither need to be frequent nor regular to be effective (Berg, 1983).

Our data support multiple independent colonizations of the Southern hemisphere and of different continents. Multiple colonizations of areas have been confirmed in the genus

*Ranunculus* in Africa (Gehrke and Linder, 2009) and in the Arctic (Hoffmann *et al.*, 2010). It seems that establishment limits distribution of taxa in an area such as the Arctic more than dispersal (Alsos *et al.*, 2007). Long distance dispersal as an important factor for distribution of taxa is recorded in other genera of Ranunculaceae as well (*Clematis*, Miikeda *et al.*, 2006; *Anemone*, Ehrendorfer *et al.*, 2009).

Although the dispersal ability of achenes has been considered limited in some *Ranunculus* species (Scherff *et al.*, 1994) there is recent evidence to suggest the contrary. Molecular phylogenetic studies indicated at least two times a colonization of Australian and New Zealand by Ranunculi against the prevailing winds (Lockhart *et al.*, 2001; Winkworth *et al.*, 2005). Achenes in buttercups do not have obvious adaptive morphological characters to disperse by wind, but Higgins *et al.*, (2003) showed that the relationship between morphological features and LDD is poor. The reason for this is that the morphology of dispersal units and the multiple processes that move seed are often complex. Various mechanisms help plants to disperse across barriers such as wind, movement by birds and animals, floating in water currents, or rafting (Thorne, 1963; Carlquist, 1966, 1967; Cain *et al.*, 2000). In general, seeds can germinate in the body of birds after two weeks (Proctor, 1968). Endozoochorous dispersal, *i.e.* in their vector's digestive system, can be also assumed for *Ranunculus* s.str. (Müller-Schneider, 1986). Indeed, viable achenes of *R. sceleratus*, an exotic species in Australia, have been collected from faecal samples of Gray Tails and successfully germinated (Green *et al.*, 2008). These birds are important aquatic and semi-aquatic plant dispersers between arid zones wetlands in Australia and can cover up to 343 km in one day (Roshier *et al.*, 2006). Moreover, local whirlwinds or transoceanic whirlpool could carry the small and light achenes of Ranunculi. Indeed, transfer of achenes by wind (anemochory), bird (ornithochory), and water (hydrochory) has been documented in species of *Ranunculus* s.str. (Müller-Schneider, 1986).

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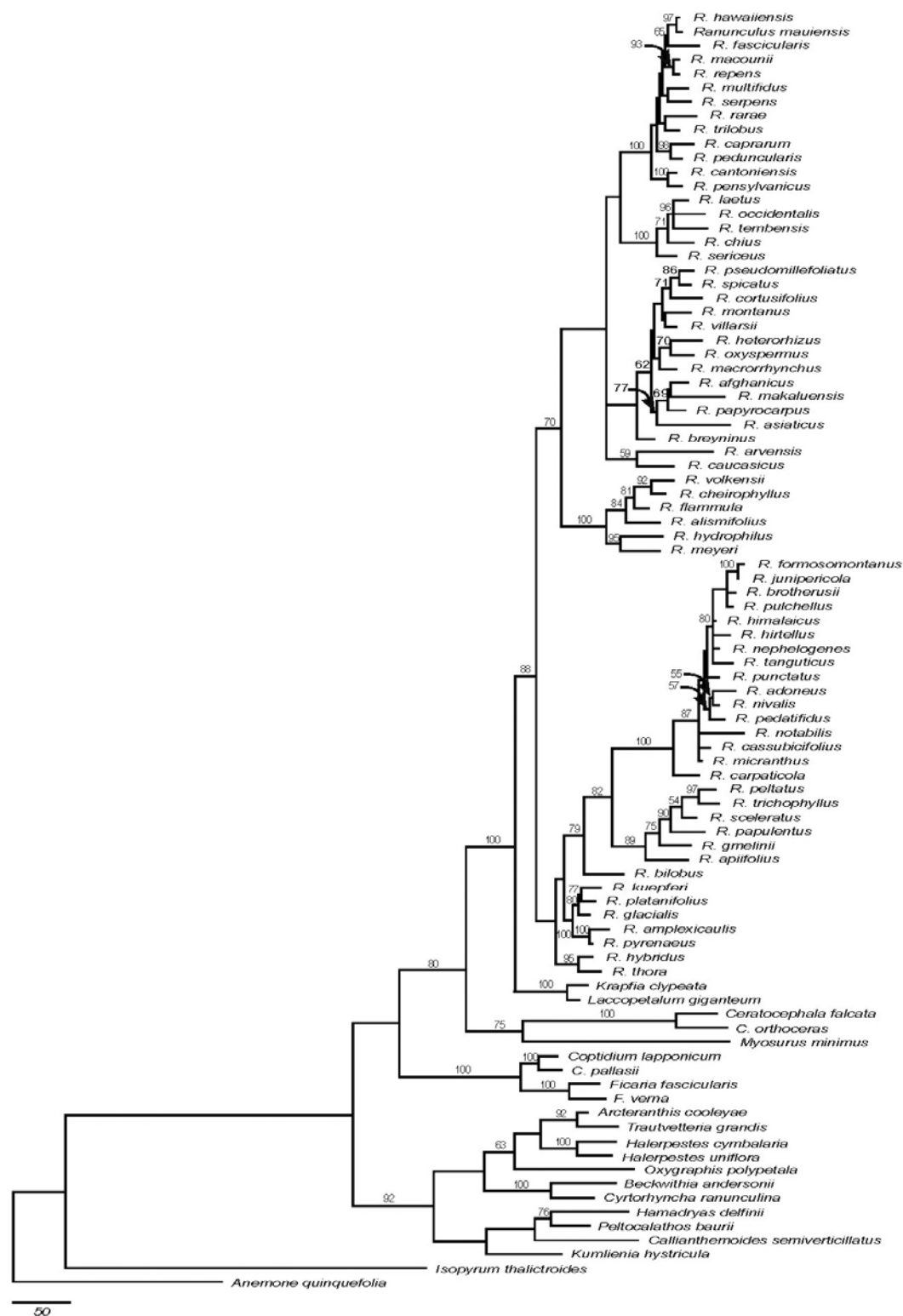
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## SUPPORTING INFORMATION

**Appendix S1** Strict consensus tree of 33 most parsimonious trees from the combined ITS, *matK*/*trnK* and *psbJ*–*petA* data set used for DIVA and Mesquite. Numbers listed above the horizontal lines are bootstrap values  $\geq 50\%$ .



**Appendix S2** Materials used in this study (BG. Botanical garden)

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Taxon (Synonym); Country; Collector, Collection number, Herbarium; ITS Genbank no.; matK/ trnK GenBank no.; psbJ-petA GenBank no.

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*Anemone quinquefolia* L.; Connecticut; Mehrhoff, 12602, CONN; GU257978; GU257980; GU257995. *Arcteranthis cooleyae* (Vasey & Rose) Greene (*R. cooleyae*); Canada; U. Jensen, 28432, MPN; AY680201; -, GU258002. *Beckwithia andersonii* (A. Gray) Jeps. (*R. andersonii*); cult. Gothenburg BG; J.T. Johansson, s.n., GB; AY680197; AY954238; GU258003. *Callianthemoides semiverticillatus* (Philippi) Tamura (*R. semiverticillatus*); Argentina; C. Lehnebach, s.n., VALD; AY680199; AY954236; Gothenburg, BG; J.T. Johansson s.n.; GU258004. *Ceratocephala falcata* (L.) Pers. (*R. falcatus*); Iran; K.H. Rechinger, Jr. 50857, W; AY680191; AY954229; GU257996. *C. orthoceras* DC. (*R. testiculatus*); Austria; E. Hörandl, 3837, WU; AY680190; AY954230; GU257997. *Coptidium lapponicum* (L.) Tzvelev (*R. lapponicus*); Sweden; J.T. Johansson, s.n., -; AY680194; AY954234; GU257998. *C. pallasii* (Schlecht.) Tzvelev (*R. pallasii*); Alaska; R. Elven et al., SUP02-175, O; AY680195; AY954233; GU257999. *Cyrthorhyncha ranunculina* Nutt. ex Torr. & A. Gray. (*R. ranunculinus*); USA; S. Nunn, 1775, RM; GU257973; GU257981; GU258005. *Ficaria fascicularis* K. Koch (*R. kochii*); cult. Gothenburg BG; Johansson, s.n., GB; AY680193; AY954231; GU258000. *F. verna* Huds. ssp. *verna* (*R. ficaria* ssp. *bulbilifer*); Sweden; Johansson, s.n., -; AY680192; AY954232; GU258001. *Halerpestes cymbalaria* (Pursh) Greene (*R. cymbalaria*); cult. Rezia BG; J.T. Johansson, 204, LD; AY680196; AY954237; GU258006. *H. uniflora* Emadzade, Lehnebach, Lockhart & Hörandl (*R. uniflorus*); Chile; C. Lehnebach, s.n., MPN; GU552270; GU552273; Argentina; M. Weigend 7003 M; GU258007. *Hamadryas delfinii* Phil.; Argentina; Schönschwetter, AR08-20, UW; GU257974; GU257982; GU258011. *Isopyrum thalictroides* L. ; Austria; E. Hörandl, 641, WU; GU257977; GU257979; GU258014. *Krapfia clypeata* (Ulbr.) Standl. & J.F. Macbr. (*R. clypeata*); Peru; Sanchez et al., 11173 F, MPN; GU552271; DQ490058; Peru; -. *Kumlienia hystricula* (A.Gray) E. Greene; USA; Hörandl, 9648, WU; GU257975; GU257983; GU258008. *Laccopetalum giganteum* Ulbr. (*R. giganteus*); Peru; Cano et al., 15196, USM; GU552272; Peru; Cano & al. 15196 USM ; DQ400695; Halle, BG; J.T. Johansson s.n.; GU258009. *Myosurus minimus* L.; ?; AJ347913; AJ414344; -. *Oxygraphis polypetala* Hook. F. & Thomson; Nepal; -, 1926-3, LI; GU257976; GU257984; GU258012. *Peltocalathos baurii* (McOwan) Tamura (*R. baurii*); South Africa; Mucina, 030103/22, WU; AY680200; AY954235; GU258010. *Ranunculus adoneus* A. Gray; USA, Colorado; Ehrendorfer, FER70, WU; AY680030; USA, Utah; Tremetsberger s.n.; \*\*\*\*\*; \*\*\*\*\*. *R. afghanicus* Aitch. & Hemsl.; Iran; Emadzade, 114, WU; \*\*\*\*\*; \*\*\*\*\*; \*\*\*\*\*. *R. alismifolius* Geyer ex Benth.; USA; Hörandl, 9651, WU; \*\*\*\*\*; \*\*\*\*\*; \*\*\*\*\*. *R. amplexicaulis* L.; cult. Lund BG; Johansson, 222, LD; AY680071; AY954223; \*\*\*\*\*. *R. apiifolius* Pers. (*Aphanostemma apiifolia*); Chile; Lehnebach s.n. VALD; AY680092; AY954140; Uruguay; Lorentz 533 W; GU258016. *R. asiaticus* L.; Iran; Shooshtari, 2569, TARI; GU257963; GU257985; GU258018. *R.*

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*bilobus* Bertol.; Italy; Hörandl, 4574, WU; AY680077; AY954220; \*\*\*\*\*. *R. breyninus* Cr. (*R. oreophilus*); Austria (loc. class.); Hörandl, 5249, WU; AY680115; AY954172; GU258021. *R. brotherusii* Freyn; Nepal; Hörandl & Emadzade, 9678, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. cantoniensis* DC; Taiwan; Huang, 1975, HAST; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. caprarum* Skottsb.; Chile, Juan Fernandez Isl.; Landero, 9355, OS; AY680151; \*\*\*\*\*, \*\*\*\*\*. *R. carpaticola* Soó; Slovakia; Hörandl, 8483, WU; AY680041; AY954111; \*\*\*\*\*. *R. cassubicifolius* W. Koch; Germany; Hörandl, 8477, WU; AY680040; AY954112; \*\*\*\*\*. *R. caucasicus* MB; Georgia; Hörandl, 8259, WU; AY680178; AY954192; GU258023. *R. cheirophyllus* Hayata; Taiwan; Hörandl, 9550, WU; GU257965; GU257987; GU258024. *R. chius* DC; Greece; Gutermann et al., 34758, WU; AY680176; AY954201; \*\*\*\*\*. *R. cortusifolius* Willd; Portugal, Madeira; Hörandl, 9586, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. fascicularis* Muhl. USA Pennsylvania; Keener, 2004-1, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. flammula* L.; cult. Oldenburg BG; Johansson, 193, CONN; AY680185; AY954204; GU258025. *R. formosomontanus* Ohwi; Taiwan; Hörandl, 9548, WU; GU257966; GU257988; GU258026. *R. glacialis* L.; Sweden; J.T. Johansson, s.n., -; AY680082; AY954219; GU258027. *R. gmelinii* ssp. *gmelinii* DC; U.S.A., Alaska; Schröck 454907, LI; AY680063; AY954128; \*\*\*\*\*. *R. hawaiiensis* A. Gray; USA; Jeffery 650079 BISH; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. heterorhizus* Boiss. & Bal; Turkey; Nydegger, 46083, M; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. ficariifolius* H. Leveille & Vaniot; Nepal; Hörandl & Emadzade, 9689, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. hirtellus* Royle Nepal; Tod; 372997, LI; AY680038; AY954120; \*\*\*\*\*. *R. hybridus*; Biria cult. Gothenburg BG; Johansson s.n., GB; AY680189; AY954211; \*\*\*\*\*. *R. hydrophilus* Gaudich.; Argentina; Schönschwetter, AR08-10, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. junipericola* Ohwi; Taiwan; Hörandl, 9547, WU ; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. kuepferi* ssp. *orientalis* W. Huber; Austria; Hörandl, 4336, WU; AY680085; AY954213; GU258028. *R. laetus* Wallich ex D.Don; India; Lone, 1750, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. macounii* Britton; Canada; Alsos & Brysting, CA72; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. macrorrhynchus* Boiss.; Iran; Emadzade, 108, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. makaluensis* Kadota; Nepal; Hörandl & Emadzade, 9700, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. mauiensis* A. Gray; USA; Oppenheimer, 684216, WU; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. meyeri* Harv.; South Africa; Gehrke et al., BG-Af 463, ZH; EU288400 EU288374; \*\*\*\*\*. *R. micranthus* Nutt.; U.S.A., Ohio; Lonsing, 50563, LI; AY680042; AY954113; \*\*\*\*\*. *R. montanus* Willd.; Austria; Hörandl, 666, WU; AY680094; AY954149; \*\*\*\*\*. *R. multifidus* Forssk.; South Africa; Mucina, 031102/7, WU; AY680162, AY954183; \*\*\*\*\*. *R. serpens* ssp. *nemorosus* (DC.) G. Lopez Gonzalez (*R. nemorosus*); Austria; Hörandl, 9522, WU; AY954243; AY954184; \*\*\*\*\*. *R. longicaulis* ssp. *nephelogenes* Edgew.; Pamir; Dikore, 17912, ?; \*\*\*\*\*, \*\*\*\*\*, \*\*\*\*\*. *R. nivalis* L.; Sweden; J.T. Johansson s.n.; AY680046; AY954123; GU258032. *R. notabilis* Hörandl & Guterm.; Austria; Hörandl, 5612, WU; AY680033; AY954115; \*\*\*\*\*. *R. occidentalis* Nutt., USA; J. Pykälä & Norris, 1139, W; *R. oxyspermus* Willd.; Iran; Emadzade, 100, WU; GU257967; GU257989; GU258033. *R. papulentus* Melville; cult. Canberra BG; Johansson, 760141p, -; AY680058; AY954138; \*\*\*\*\*. *R.*

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*papyrocarpus* Rech. F., Aell. & Esfand.; Iran; Tajeddini, 110, WU; GU257968; GU257990; GU258034. *R. pedatifidus* J.E. Smith; USA; Orthner, 593RM; GU257969; GU257991; GU258036. *R. peduncularis* Sm.; Chile; Lehnebach s.n., VALD; AY680154; AY954180; Argentina; Schönschwetter, Ar08-23, WU; \*\*\*\*\*. *R. peltatus* ssp. *peltatus* Moench (*Batrachium peltatum*); cult. Nantes BG; J.T. Johansson 206 LD; AY680068; AY954131; \*\*\*\*\*. *R. pensylvanicus* L. f.; U.S.A.; V. Zila 447002 LI; AY680147; AY954190; GU258038. *R. platanifolius* L.; Norway; Johansson, 277, LD; AY680080; AY954216; \*\*\*\*\*. *R. pseudomillefoliatus* Grau; Spain; Schneeweiss et al., 7253, WU; AY680110; AY954156; \*\*\*\*\*. *R. pulchellus* C.A.Mey; Nepal; Hörandl & Emadzade, 9679, WU; \*\*\*\*\*; \*\*\*\*\*; \*\*\*\*\*. *R. punctatus* Jurtzev; Russia; Zimarskaya et al., s.n., LE; FM242818; FM242754; \*\*\*\*\*. *R. pyrenaeus* L.; Spain; Schneeweiss et al., 6498, WU; AY680074; AY954225; GU258041. *R. rarae* Exell; Malawit; Gehrke et al., BG-Af 304, ZH; EU288416; EU288389; \*\*\*\*\*. *R. repens* L.; Iran; Emadzade, 107, WU; \*\*\*\*\*; \*\*\*\*\*; \*\*\*\*\*. *R. sceleratus* L.; Iran; Emadzade, 112, WU; GU257971; GU257993; GU258043. *R. sericeus* Banks & Soland; Iran, Emadzade, 121, WU; \*\*\*\*\*; \*\*\*\*\*; \*\*\*\*\*. *R. spicatus* Desf.; cult. Wisley BG; Johansson s.n., LD; AY954244; AY954158; \*\*\*\*\*. *R. tembensis* Hochst. ex A. Rich.; Ethiopia; Gehrke et al., BG-Af 210, ZH; EU288421; EU288393; \*\*\*\*\*. *R. thora* L.; cult. Lund BG; Johansson, 223, LD; AY680188; AY954210; GU258045. *R. trichophyllus* Chaix (*Batrachium trichophyllum*); Greece; Dahlgren, B23, LD; AY680067; AY954133; GU258046. *R. trilobus* Desf.; cult. Antwerpen BG; Johansson, 217, LD; AY680149; AY954176; \*\*\*\*\*. *R. villarsii* DC. (*R. grenieranus*); Austria; Hörandl, 664, WU; AY680099; AY954153; \*\*\*\*\*. *R. volkensii* Engl.; Uganda; Gehrke et al., BG-Af353, ZH; EU288424; EU288396; \*\*\*\*\*. *Trautvetteria grandis* Honda; cult. California BG; J.T. Johansson 82.1322 -; AY680202; AF007945; GU258013, *Xanthorhiza simplicissima* Marshall; Genbank; -; AB069848;-.

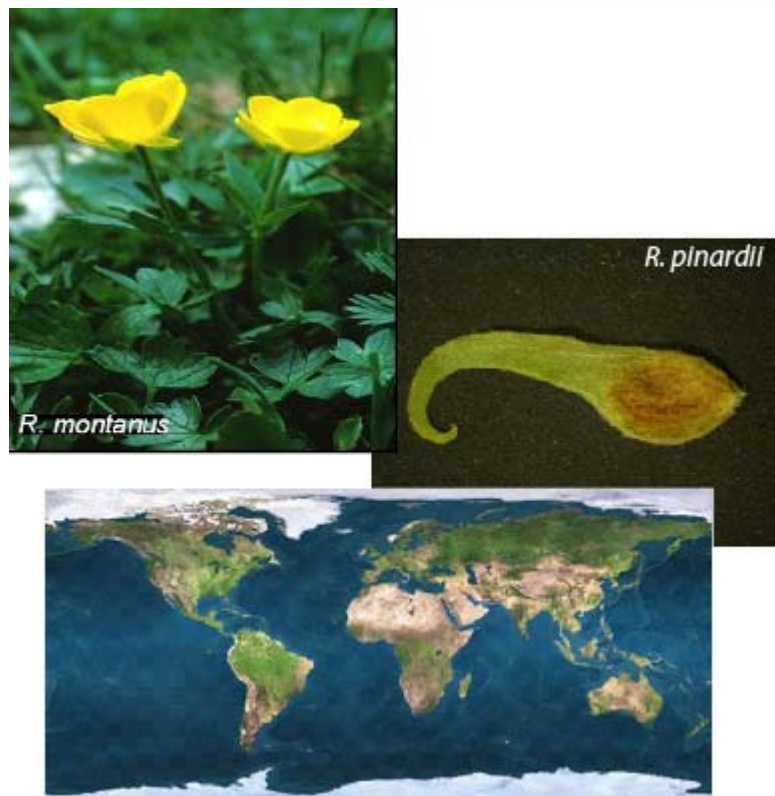
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\*\*\*\*\* Sequences which will be submitted to GenBank for publishing.

## Chapter 4

### The biogeographical history of the cosmopolitan genus *Ranunculus* L. (Ranunculaceae) in the temperate to meridional zones\*

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## ABSTRACT

*Ranunculus* is distributed in all continents and especially species-rich in the meridional and temperate zones. To reconstruct the biogeographical history of the genus, a molecular phylogenetic analysis of the genus based on nuclear and chloroplast DNA sequences has been carried out. Results of biogeographical analyses (DIVA, Lagrange, Mesquite) combined with molecular dating suggest multiple colonizations of all continents and disjunctions between the northern and the southern hemisphere. Dispersals between continents must have occurred via migration over land bridges, or via transoceanic long-distance dispersal, which is also inferred from island endemism. In southern Eurasia, isolation of the western Mediterranean and the Caucasus region during the Messinian was followed by range expansions and speciation in both areas. In the Pliocene and Pleistocene, radiations happened independently in the summer-dry W. Mediterranean-Makaronesian and in the E. Mediterranean-Irano-Turanian regions, with three independent shifts to alpine humid climates in the Alps and in the Himalayas. The cosmopolitan distribution of *Ranunculus* is caused by transoceanic and intracontinental dispersal, followed by regional adaptive radiations.

**Keywords:** *Ranunculus*, molecular phylogenetics, biogeographical history, dispersal, vicariance

## 1. Introduction

*Ranunculus* s.str. is a cosmopolitan genus with approximately 600 species (Tamura, 1993, 1995) and the largest genus in Ranunculaceae. *Ranunculus* is distributed on all continents and it has a worldwide distribution from the Tropics to the arctic and subantarctic zones. The genus is especially species-rich in temperate to meridional zones (e.g., Ovczinnikov, 1937; Iranshahr *et al.* 1992; Whittemore, 1997). In the tropical areas, species are restricted to high mountain areas (e.g. African species; Tamura, 1993, 1995). Species of *Ranunculus* are established in a variety of wet to dry habitats from the lowland to high alpine zones and show several morphological adaptations to different habitats (Paun *et al.*, 2005; Emadzade *et al.*, in prep.). In mountain areas, endemism contributes to the considerable species diversity, but in lower altitudes widespread species are also quite common. *Ranunculus* shows different levels of polyploidy, which is sometimes connected to apomixis (Hörandl *et al.*, 2005).

Monophyly of *Ranunculus* has been assumed by previous molecular phylogenetic studies (Hoot, 1994; Johansson, 1995, 1998; Ro *et al.*, 1997; Hörandl *et al.*, 2005; Paun *et al.*, 2005; Lehnebach *et al.*, 2007; Gehrke and Linder, 2009; Hoffmann *et al.*, 2010; Emadzade *et al.*, in press). Previous studies (using cpDNA restriction sites, Johansson, 1998; ITS sequences, Hörandl *et al.*, 2005; matK/trnK plus ITS, Paun *et al.*, 2005; Lehnebach, 2008; Gehrke and Linder 2009; Hoffmann *et al.*, 2010) showed that the core *Ranunculus* clade was subdivided into several well-supported clades that corresponded to widespread ecological groups (e.g., wetland and aquatic species) or to regional geographical groups (e.g., in the European mountain system; Hörandl *et al.*, 2005; Paun *et al.*, 2005). Biogeographical studies focusing on certain areas suggested multiple colonizations of Africa (Gehrke and Linder 2009) and of the Arctic (Hoffmann *et al.*, 2010). However, the biogeographical processes that have shaped the global distribution of buttercups are still not well understood. The frequently observed intercontinental disjunctions in earlier studies could be due to wide distributions of the ancestors that have been separated via geographical barriers, followed by allopatric speciation and diversification (vicariance). Alternatively, the lineages within the clades had the ability for dispersal via seeds or propagules to new areas, followed by speciation and adaptation to new habitats. Endemism on oceanic islands like Hawaii, Juan Fernandez Islands, and Macaronesia is another indication for the high ability of buttercups for long-distance dispersal (LDD), speciation and rapid adaptation to new habitats. A northern hemispheric origin followed by vicariance and transoceanic dispersal has shaped the distributional patterns in Ranunculeae (Emadzade and Hörandl, submitted). These genera, however, are not as

diversified and widely distributed as *Ranunculus* s.str.; some of them are monotypic and regional endemics. In contrast, *Ranunculus* s.str., shows not only the ability for long-distance dispersal to new areas, but also a potential for adaptive radiations (Lockhart *et al.* 2001; Paun *et al.* 2005).

The origin of *Ranunculus* probably dates back to the late Oligocene, and was followed by several waves of diversification until the Quaternary (Paun *et al.*, 2005; Hoffmann *et al.*, 2010; Emadzade and Hörandl, submitted). The high number of species and endemism, the global distribution, and the observed temporal and spatial patterns make this genus interesting for studying historical biogeography. However, previous studies focused only on certain regions (Mediterranean, Paun *et al.*, 2005; Africa, Gehrke and Linder, 2009; Arctic, Hoffmann *et al.*, 2010) and did not apply analytical tools of biogeography. The biogeographical history of related genera has been presented elsewhere (Emadzadeh and Hörandl, subm.). A comprehensive biogeographical analysis of the cosmopolitan genus *Ranunculus* s.str. based on a worldwide sampling was so far missing.

Based on a molecular phylogenetic reconstruction (Fig. 1), we focus here on a species-rich clade comprising mainly species of the temperate to the meridional zones (Fig. 1a, clades V-IX). We did not attempt a reconstruction of the biogeographical history of *Ranunculus* as a whole, because, at first, the backbone phylogeny is not well resolved, and relationships of big clades (I-IX) to each other are not well supported (Fig. 1); second, the clades with meridional-temperate (V-IX) species showed a better resolution compared to the high alpine, arctic or wetland groups (Fig. 1b, clades I-IV). Previous molecular dating approaches (Paun *et al.*, 2005, Hoffman *et al.*, 2010; Emadzade and Hörandl, submitted) suggested origin and diversification of the meridional-temperate clades already in the Miocene. Because of the age and the southern distribution, the spatial-temporal diversification in these clades was not so much influenced by range fluctuations and extinctions due to Quaternary glaciations. The meridional to temperate clades comprise species from all continents (except Antarctica), which allows for the analysis of intercontinental disjunctions, dispersal, and vicariance events between continents in a global framework. Previous phylogenetic studies suggest that the temperate zone was the source area for both subtropical-tropical and arctic species (Gehrke and Linder, 2009; Hoffmann *et al.*, 2010). The biogeographical processes in the meridional to temperate zones are therefore of crucial importance for understanding the biogeographical history of the genus. The species richness in these areas further raises the question whether intracontinental dispersal and regional radiations have played a role for the diversity and wide

distribution of the genus. For this question, the biogeographical patterns in southern Eurasia can serve as a model system for other continents. This “ancient Tethyan area” (Takhtajan, 1986) is of special interest for biogeographical questions because of its complex geological and climatic history. In the Mediterranean area, sea-level fluctuations, including desiccation and later re-flooding of the Mediterranean sea, establishment of a summer-dry climate and the uplift of mountain chains have caused both geographic and eco-climatological differentiation processes in flowering plants (Thompson, 2005; Lo Presti and Oberprieler, 2009). The direct geographical connection of the Mediterranean to the Irano-Turanian region, and the continuation of the European Alpine system in the Central Asian mountain chains provide migration routes and have formed a distinct biogeographical area (“ancient Tethyan area” sensu Takhtajan 1986).

In contrast, the alpine-arctic (Fig. 1, clades I, II, IV) and the wetland clades (clade III) have been influenced by reticulate evolution, hybridization and high frequencies of polyploids which is problematic for tree-based biogeographical analyses because of a non-hierarchical structure of data (Lockhart *et al.*, 2001; Hörandl *et al.*, 2005, 2009). In clades I, II, and III, our analyses confirmed geographical patterns of previous studies (Hörandl *et al.*, 2005; Paun *et al.*, 2005): clades I and II comprise mainly European alpine species, while clade III consists of widespread wetland or aquatic species. The evolutionary history of the alpine-arctic clade (Fig. 1b, clade IV) will be presented elsewhere (Emadzade *et al.* in prep).

Molecular phylogenetic data, including molecular age estimates, provide a strong hypothesis for understanding the biogeographical history of the temperate-meridional species. We combine here the results from previous molecular dating studies (Paun *et al.*, 2005; Hoffmann *et al.* 2010; Emadzade and Hörandl, submitted) and biogeographical analyses 1) to provide a comprehensive hypothesis of the history of *Ranunculus* in the meridional to temperate zones in a global context, 2) to develop hypotheses for the spatial distribution of buttercups in the context of the geological history of the different continents, 3) to investigate the main migration routes between continents and areas of diversity, 4) to reconstruct the main factor(s) shaping the modern distribution of the genus, including the relative role of long-distance dispersal and vicariance. Additionally, we reconstruct the main processes that have caused the modern distribution and diversity of taxa in greater detail in the “Tethyan” clade, Fig. 1a, clade IX) comprising species from the whole Mediterranean-Makaronesian area, the Circumboreal area, the Irano-Turanian region, Central Asia, and the Himalayas.

## 2. Materials and methods

### 2.1. Plant material

We sampled 185 species of *Ranunculus* s.str. (Tamura, 1993, 1995) and 20 species of allied genera to develop a basic phylogenetic framework. This collection covers more than one third of the buttercups from all continents except Oceania from where samples were not available. *Anemone* and *Isopyrum* were used as outgroup taxa. A nuclear marker (the ITS region of the nuclear ribosomal DNA) and two chloroplast markers (*matK/trnK*) were obtained from 71 new species and combined with data from previous studies (Hörandl *et al.*, 2005; Paun *et al.*, 2005; Gehrke and Linder, 2009; Hoffmann *et al.*, 2010). The *psbJ-petA* region was newly sequenced for all species. We used only samples for which sequences of all markers were available. Voucher information and GenBank accession numbers are provided in Table 1.

### 2.2. DNA extraction, amplification, and sequencing

Total genomic DNA from silica-dried or herbarium material was extracted using a modified CTAB technique (Doyle and Doyle, 1987). The whole internal transcribed spacer region (ITS, including ITS1, the 5.8 gene, ITS2) was amplified as a single piece with primers ITS 18sF and ITS 26sR (Gruenstaeudl *et al.*, 2009) or in the case of degraded DNA from poor quality herbarium tissue, in two pieces with additional primers (ITS 5.8sF and ITS 5.8sR) as internal primers (Gruenstaeudl *et al.*, 2009). Sequencing of the *matK/trnK* region was performed according to the protocol described by Paun *et al.* (2005). Amplification of the non coding *PsbJ/PetA* region was carried out as a single piece in all samples by using primers of Shaw *et al.* (2007). PCR was performed in 23 µl reactions containing 20 µl 1.1× Reddy Mix PCR Master Mix (including 2.5 mM MgCl<sub>2</sub>; ABgene, Epsom, UK), 1 µl of 0.4% bovine serum albumin (BSA, Promega, Madison, WI, U.S.A.), and in the case of the ITS region, dimethyl sulfoxide (DMSO) to reduce problems associated with DNA secondary structure, 1 µl each primer (10 mmol/L) and 1 µl template DNA. PCR products were purified using *E. coli* Exonuclease I and Calf Intestine Alkaline Phosphate (CIAP; MBI-Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Cycle sequencing was performed using Big Dye<sup>TM</sup> Terminator v3.1 Ready Reaction Mix (Applied Biosystems), using the following cycling conditions: 38 cycles of 10 sec at 96°C, 25 sec at 50°C, 4 min at 60°C. All DNA regions were sequenced in both directions. The samples were run on a 3130xl Genetic Analyzers capillary sequencer (Applied Biosystems).

**Table 1** Species sampled, voucher information and GenBank accessions of DNA sequences analyses in this study (BG: Botanical garden).

Taxon	Country; collector, collection No.; Herbar	GenBank accession Nos.		
		ITS	<i>matK/trnK</i>	<i>psbJ-petA</i>
<i>Anemone quinquefolia</i> L.	Connecticut; Mehrhoff 12602; CONN	GU257978	GU257980	GU257995
<i>Arcteranthus cooleyae</i> (Vasey & Rose) Greene	Canada; Jensen 28432; MPN	AY680201	-	GU258002
<i>Beckwithia andersonii</i> (A. Gray) Jeps.	cult. Gothenburg BG; Johansson s.n.; GB	AY680197	AY954238	GU258003
<i>Callianthemoides semiverticillatus</i> (Philippi) Tamura	Argentina; Lehnbach s.n.; VALD	AY680199	AY954236	Gothenburg, BG; Johansson s.n.; GU258004
<i>Ceratocephala falcata</i> (L.) Pers.	Iran; Rechinger Jr.50857; W	AY680191	AY954229	GU257996
<i>C. orthoceras</i> DC.	Austria; Hörandl 3837; WU	AY680190	AY954230	GU257997
<i>Coptidium lapponicum</i> (L.) Tzvelev	Sweden; Johansson s.n.; -	AY680194	AY954234	GU257997
<i>C. pallasi</i> (Schlecht.) Tzvelev	Alaska; Elven & al. SUP02-175; O	AY680195	AY954233	GU257999
<i>Cyrtorhyncha ranunculina</i> Nutt ex. Torr. & A. Gray.	USA; Nunn 1775; RM	GU257973	GU257981	GU258005
<i>Ficaria fascicularis</i> K.Koch	cult. Gothenburg BG; Johansson s.n.; GB	AY680193	AY954231	GU258000
<i>F. verna</i> Huds. ssp. <i>verna</i>	Sweden ; Johansson s.n. -;	AY680192	AY954232	GU258001
<i>Halerpestes cymbalaria</i> (Pursh) Greene	cult. Rezia BG; Johansson 204; LD	AY680196	AY954237	GU258006
<i>H. uniflora</i> (Phil. ex. Reiche) Emadzade <i>et al.</i>	Chile; Lehnbach s.n.; MPN	GU552270	GU552273	Argentina; Weigend 7003; M; GU258007
<i>Hamadryas delfinii</i> Phil.	Argentina; Schönswetter AR08-20; WU	GU257974	GU257982	GU258011
<i>Isopyrum thalictroides</i> L.	Austria; Hörandl 641; WU	GU257977	GU257979	GU258014
<i>Krapfia clypeata</i> (Ulbr.) Standl. & J.F.Macbr.	Peru; Sanchez & al. 11173 F, CPUN, MPN	GU552271	DQ490058	-
<i>Kumlienia hystrix</i> (A.Gray) E. Greene	USA; Hörandl 9648; WU	GU257975	GU257983	GU258008
<i>Laccopetalum giganteum</i> Ulbr.	Halle, BG; J.T. Johansson s.n.;	GU552272	Peru; Cano & al. 15196; USM DQ400695	Halle, BG; J.T. Johansson s.n.;; GU258009
<i>Myosurus minimus</i> L.	Genbank	AJ347913	AJ414344	-
<i>Oxygraphis polypetala</i> Hook. F. & Thomson.	Nepal; - 1926-3; LI	GU257976	GU257984	GU258012
<i>Peltocalathos baurii</i> (McOwan) Tamura	South Africa; Mucina 030103/22; WU	AY680200	AY954235	GU258010
<i>R. acetosellifolius</i> Boiss.	cult. Gothenburg BG ; Johansson s.n. ;—	AY680075	AY954226	+
<i>R. aconitifolius</i> L.	cult. Copenhagen BG ; Johansson 274; LD	AY680081	AY954217	+
<i>R. acrifolius</i> A. Gray	USA, Utah; Albach 844; WU	+	+	+
<i>R. acris</i> L.	cult. Bonn BG ; Johansson 194; CONN	AY680167	AY954199	GU258015
<i>R. adoneus</i> A. Gray	USA, Colorado; Ehrendorfer FER70; WU	AY680030	+	USA, Utah; Tremetsberger s.n.; WU; +
<i>R. aduncus</i> Gren. & Godr.	Italy; Hörandl 6818; WU	AY680088	AY954143	+
<i>R. afghanicus</i> Aitch. & Hemsl.	Iran; Emadzade 114; WU	+	+	+
<i>R. alismifolius</i> Geyer ex Benth.	USA; Hörandl 9651; WU	+	+	+
<i>R. alpestris</i> L.	cult. Rezia BG; Johansson 242; LD	AY680078	AY954221	+
<i>R. amblyolobus</i> Boiss. & Hohen.	Iran; Emadzade 120; WU	+	+	+
<i>R. amplexicaulis</i> L.	cult. Lund BG; Johansson 222; LD	AY680071	AY954223	+
<i>R. apenninus</i> (Chiov.) Pign.	Italy; Hörandl 6069; WU	AY680091	AY954150	+
<i>R. apiifolius</i> Pers. ( <i>Aphanostemma apiifolia</i> )	Chile; Lehnbach s.n.; VALD	AY680092	AY954140	Uruguay; Lorentz 533 W; GU258016
<i>R. aquatilis</i> L.	USA; Hörandl 9625; WU	+	+	+
<i>R. argyreus</i> Boiss.	Turkey; Brause 45; LE	FM242844	FM242780	+
<i>R. arvensis</i> L.	cult. Kiel BG; Johansson 180; CONN	AY680177	AY954193	Iran; Emadzade 109; WU; GU258017
<i>R. asiaticus</i> L.	Iran; Shooshtari 2569; TARI	GU257963	GU257985	GU258018
<i>R. aucheri</i> Boiss.	Iran; Emadzade 101; WU	+	+	+
<i>R. baldschuanicus</i> Regel ex Kom.	cult. Copenhagen BG; Johansson 272; LD	AY680174	AY954195	+
<i>R. bilobus</i> Bertol.	Italy; Hörandl 4574; WU	AY680077	AY954220	+
<i>R. bonariensis</i> Poir.	Argentina; Schönswetter AR08-2a; WU	GU257964	GU257986	GU258019
<i>R. brachylobus</i> Boiss. & Hohen.	Iran; Emadzade 115; WU	+	+	+
<i>R. brevifolius</i> ssp. <i>brevifolius</i> Ten.	cult. Gothenburg BG; Johansson s.n.; GB	AY680187	AY954212	GU258020
<i>R. breynius</i> Cr.	Austria; Hörandl 5249; WU	AY680115	AY954172	GU258021
<i>R. brotherusii</i> Freyn	Nepal; Hörandl & Emadzade 9678; WU	+	+	+
<i>R. brutius</i> Tenore	Italy; Pittoni s.n.; M	+	+	+
<i>R. buhsei</i> Boiss.	Russia; Ahms -; HAL	FM242860	FM242796	+
<i>R. bulbosus</i> ssp. <i>bulbosus</i> L.	Sweden; Johansson s.n.; -	AY680124	AY954188	+
<i>R. bullatus</i> L.	Greece; Hörandl & Gutermann 7191; WU	AY680114	AY954161	+
<i>R. cacuminis</i> Strid & Papan.	Greece; Huberk & Krug 13565; Z & ZT	+	+	+
<i>R. camissonis</i> Aucl.	USSR; Koropewa s.n.; W	AY680083	AY954218	GU258022
<i>R. cantoniensis</i> DC.	Taiwan; Huang 1975 ; HAST	+	+	+

<i>R. cappadocicus</i> Willd.	Georgia, Kaukasus; Hörandl 8269; WU	AY680117	AY954173	+
<i>R. caprarum</i> Skottsbo.	Chile; Juan Fernandez Isl., Landerø 9355; OS	AY680151	+	+
<i>R. cardiophyllus</i> Hook.	cult. Gothenburg BG; Johansson HZ 86-29, GB	AY680045	AY954124	+
<i>R. carinthiacus</i> Hoppe	Austria; Hörandl 4096; WU	AY680093	AY954145	+
<i>R. carpaticola</i> Soó	Slovakia; Hörandl 8483; WU	AY680041	AY954111	FJ619866
<i>R. carpaticus</i> Herbach	Romania; Paun s.n.; WU	AY680096	AY954154	+
<i>R. cassius</i> Boiss.	Lebanon; Maitland 289; LE	FM242848	FM242784	+
<i>R. cassubicifolius</i> W. Koch	Germany; Hörandl 8476; WU	AY680040	AY954112	FJ619867
<i>R. caucasicus</i> MB.	Georgia; Hörandl 8259; WU	AY680178	AY954192	GU258023
<i>R. cheiropphyllus</i> Hayata	Taiwan; Hörandl 9550; WU	GU257965	GU257987	GU258024
<i>R. chinensis</i> Bunge	Russia; Khakevich & Buch 1355; ZT	+	+	+
<i>R. chius</i> DC.	Greece; Gutermann & al. 34758; WU	AY680176	AY954201	+
<i>R. cicutarius</i> Schlecht.	Iran; Akhani 320156; LI	AY680103	AY954167	+
<i>R. collinus</i> DC.	cult. Canberra BG; Crisp & Telford 2227; CAN	AY680059	AY954137	+
<i>R. constantinopolitanus</i> (DC.) d'Urv.	Iran; Memarianii 117; WU	+	+	+
<i>R. cornutus</i> DC.	Azerbaijan; Schneeweiss 6806; WU	AY680153	AY954178	+
<i>R. cortusifolius</i> Willd.	cult. Halle BG; Johansson 237 ;LD	AY680101	AY954160	+
<i>R. crenatus</i> Waldst. & Kit.	Austria; Hörandl 2818; WU	AY680086	AY954228	+
<i>R. damascenus</i> Boiss. & Gaill.	Turkey; Nydegger 41126; ZT	+	+	+
<i>R. diffusus</i> DC.	Nepal; Hörandl & Emadzade 9706; WU	+	+	+
<i>R. dissectus</i> M. Bieb. var. <i>napellifolius</i> (DC.) P.H. Davis	Turkey; Walther 9258; LE	FM242849	FM242785	+
<i>R. elbursensis</i> Boiss.	Iran; Emadzade 105; WU	+	+	+
<i>R. eschscholtzii</i> Schlecht.	Canada; Jensen UJ8; MPN	AY680050	AY954127	USA; Albach 838; WU; +
<i>R. fascicularis</i> Muhl.	USA Pennsylvania; Keener 2004-1; WU	+	+	+
<i>R. flagelliformis</i> Sm.	Peru; Gute & Muler 309853; LI	AY680182	AY954208	+
<i>R. flammula</i> L.	cult. Oldenburg BG; Johansson 193; CONN	AY680185	AY954204	GU258025
<i>R. ficarifolius</i> Leveill & Van	Nepal; Hörandl & Emadzade 9677b; WU	+	+	+
<i>R. fuegianus</i> Speg.	Chile; L. & F. Ehrendorfer s.n.; VALD	AY680064	AY954136	Argentina; Schönschwetter Ar08-14; WU; +
<i>R. garganicus</i> Ten.	Greece; Gutermann & al. 34974; WU	AY680107	AY954165	+
<i>R. gelidus</i> Kar. & Kir.	Xinjiang, China; Wang 28426; MPN	AY680054	AY954114	+
<i>R. glabriusculus</i> Rupr.	Russia; Skvortsov 10913; M	+	+	+
<i>R. glacialis</i> L.	Sweden; Johansson s.n. -	AY680082	AY954219	GU258027
<i>R. gmelinii</i> ssp. <i>gmelinii</i> DC.	U.S.A., Alaska; Schröck 454907; LI	AY680063	AY954128	+
<i>R. gouanii</i> Willd.	cult. Schachen; Johansson s.n. -	AY680098	AY954151	+
<i>R. gracilis</i> Schlecht.	Greece; Johansson s.n. -	AY680120	AY954171	+
<i>R. gramineus</i> L.	cult. Krefeld BG; Johansson s.n. -	AY680076	AY954227	+
<i>R. granatensis</i> Boiss.	unknown; Johansson 266; LD	AY680165	AY954197	+
<i>R. grandiflorus</i> L.	Georgia; Hörandl 8271; WU	AY680053	AY954203	+
<i>R. gregarius</i> Brot.	cult. Berlin-Dahlem BG; Johansson 232; LD	AY680100	AY954159	+
<i>R. hawaiiensis</i> A. Gray	USA; Jeffery 650079; BISH	+	+	+
<i>R. heterorhizus</i> Boiss. & Bal.	Turkey ;Nydegger; 46083; M	+	+	+
<i>R. hierosolymitanus</i> Boiss.	Palestina; Favrat s.n. ; ZT	+	+	+
<i>R. hirtellus</i> Royle	Nepal; Tod 372997; LI	AY680038	AY954120	+
<i>R. hispidus</i> Michx.	USA, Pennsylvania; Keener 2004-3b; WU	+	+	+
<i>R. hybridus</i> Bria	cult. Gothenburg BG; Johansson s.n. GB	AY680189	AY954211	+
<i>R. hydrophilus</i> Gaudich.	Argentina; Schönschwetter AR08-10; WU	+	+	+
<i>R. hyperboreus</i> Rottb.	Sweden; Johansson s.n. -	AY680065	AY954135	+
<i>R. illyricus</i> L.	Sweden; Lundgren s.n.; -	AY680119	AY954162	+
<i>R. japonicus</i> Thunb.	China; XieLei XL200348; WU	AY680164	AY954200	+
<i>R. kotschy</i> Boiss.	Iran; Emadzade 113; WU	+	+	+
<i>R. kuepferi</i> Greuter & Burdet	Austria; Hörandl 4336; WU	AY680085	AY954213	GU258028
<i>R. laetus</i> Wallich ex D.Don	India; Lone 1750; WU	+	+	+
<i>R. laetus</i> Wallich ex D.Don	India; Lone 1761; WU	+	+	+
<i>R. lanuginosus</i> L.	Unknown; Johansson 255; LD	AY680163	AY954194	+
<i>R. lateriflorus</i> DC.	cult. Catania BG; Johansson 235; LD	AY680179	AY954209	+
<i>R. leptorrhynchus</i> Aitch. & Hemsl.	Iran; Emadzade 111; WU	+	+	+
<i>R. linearilobus</i> Bunge	Afghanistan; Podlech 10374; M	+	+	+
<i>R. lingua</i> L.	cult. Lund BG; Johansson s.n.; -	AY680184	AY954206	+
<i>R. longicaulis</i> C.A.Mey.	Pakistan; Millinger 470564; LI	AY680051	AY954117	+
<i>R. lyallii</i> Hook. f.	New Zealand; Steel 24603; MPN	AF323277	AY954142	+
<i>R. maclovianus</i> Urv.	Chile; Lehnebach s.n.; VALD	AY680158	AY954181	+
<i>R. macounii</i> Britton	Canada; Alsos & Brysting CA72;?	+	+	+
<i>R. macropodioides</i> Briq.	Iran; Mozaffarian 77929; TARI	+	+	+
<i>R. macrorrhynchus</i> Boiss.	Iran; Emadzade 108; WU	+	+	+
<i>R. magellensis</i> Ten.	Italy; Baltisberger & Krug 12831; Z & ZT	+	+	+
<i>R. makaluensis</i> Kadota	Nepal; Hörandl & Emadzade 9700; WU	+	+	+

<i>R. marginatus</i> Urv.	cult. Copenhagen BG; Johansson 286; LD	AY680150	AY954177	+
<i>R. marschlinii</i> Steud.	Corse; Hörandl 6981; WU	AY680089	AY954147	+
<i>R. mauianensis</i> A. Gray	USA; Oppenheimer 684216; WU	+	+	+
<i>R. membranaceus</i> Royle	Nepal; Hörandl & Emadzade 9696; WU	+	+	+
<i>R. meyeri</i> Harv.	South Africa; Gehrke <i>et al.</i> BG-Af 463, ZH	EU288400	EU288374	+
<i>R. micranthus</i> Nutt.	U.S.A., Ohio; Lonsing 50563; LI	AY680042	AY954113	+
<i>R. millefoliatus</i> Vahl	cult. Graz BG; Johansson 293; LD	AY680108	AY954166	+
<i>R. millefolius</i> Banks & Soland.	Iran; Emadzade 121; WU	+	+	+
<i>R. montanus</i> Willd.	s.s. Austria; Hörandl 666; WU	AY680094	AY954149	+
<i>R. multifidus</i> Forssk.	South Africa; Mucina 031102/7; WU	AY680162	AY954183	+
<i>R. muricatus</i> L.	cult. Siena BG; Johansson 210; LD	AY680148	AY954191	+
<i>R. natans</i> C.A.Mey.	Russia; Tribsch 9558; WU	AY680113	AY954134	GU258031
<i>R. neapolitanus</i> Ten.	Greece; Johansson 224; LD	AY680123	AY954187	+
<i>R. nepelogenes</i> Edgew.	Pamir; Dickore 17912; M	+	+	+
<i>R. nipponicus</i> Nakai	Russia; Egorova s.n.; LE	FM242834	FM242770	+
<i>R. nivalis</i> L.	Sweden; Johansson s.n.; -.	AY680046	AY954123	GU258032
<i>R. notabilis</i> Hörandl & Guterm.	Austria; Hörandl 5612; WU	AY680033	AY954115	FJ619873
<i>R. occidentalis</i> Nutt.	USA; Hörandl 9644; WU	+	+	+
<i>R. olisipponensis</i> Pers.	Spain; Gutermann 37407; WU	AY680109	AY954157	+
<i>R. ophioglossifolius</i> Vill.	cult. Nantes BG J.T. Johansson 208 LD	AY680180	AY954207	+
<i>R. oreophytus</i> Delile	Ethiopia; Gehrke <i>et al.</i> BG-Af 209, ZH	EU288412	+	+
<i>R. orthorhynchus</i> Hook.	U.S.A., Hörandl 9618 WU; UT	+	+	+
<i>R. oxyspermus</i> Willd.	Iran; Emadzade 100; WU	GU257967	GU257989	GU258033
<i>R. paludosus</i> Poir.	Greece; Gutermann & al. 34754; WU	AY680102	AY954155	+
<i>R. papulentus</i> Melville	cult. Canberra BG; Johansson 760141p -	AY680058	AY954138	+
<i>R. papyrocarpus</i> Rech. F., Aell. & Esfand.	Iran; Tajeddini 110; WU	GU257968	GU257990	GU258034
<i>R. parnassifolius</i> ssp. <i>parnassifolius</i> L.	France/Spain Schneeweiss & al. 6509; WU	AY680072	AY954224	GU258035
<i>R. parviflorus</i> L.	cult. Copenhagen BG; Johansson 287; LD	AY680175	AY954202	+
<i>R. pedatifidus</i> J.E. Smith,	USA; Orthner 593; RM	GU257969	GU257991	GU258036
<i>R. peduncularis</i> Sm.	Chile; Lehnbech s.n.; VALD	AY680154	AY954180	-
<i>R. peltatus</i> ssp. <i>peltatus</i> Moench	cult. Nantes BG; Johansson 206; LD	AY680068	AY954131	GU258037
<i>R. penicillatus</i> ssp. <i>pseudofluitans</i> (Dum.) Bab.	England; G. Dahlgren BE9; LD	AY680070	AY954130	+
<i>R. pennsylvanicus</i> L.	U.S.A.; Zila 447002; LI	AY680147	AY954190	GU258038
<i>R. petiolaris</i> Kunth ex DC.	USA; Stuessy 18581; WU	+	+	+
<i>R. pinardii</i> (Stev.) Boiss.	Iran; Ghahremanii 108; WU	GU257970	GU257992	GU258039
<i>R. pinnatus</i> Poir.	Madagascar; Gehrke <i>et al.</i> BG-Af 247, ZH	EU288415	EU288388	+
<i>R. platanifolius</i> L.	Norway; Johansson 277; LD	AY680080	AY954216	+
<i>R. pollinensis</i> Chiovenda	Italy; Hörandl 8247; WU	AY680097	AY954152	+
<i>R. polyanthemos</i> L.	Austria; Hörandl 5130; WU	AY680121	AY954185	GU258040
<i>R. pseudohirculus</i> Schrenk ex F.E. Fischer & C.A. Mey.	Russia; Tribsch 9593; WU	AY680111	AY954118	+
<i>R. pseudomillefoliatus</i> Grau	Spain; Schneeweiss & al. 7253; WU	AY680110	AY954156	+
<i>R. pseudomontanus</i> Schur	Slovakia; Hörandl 5904; WU	AY680090	AY954146	+
<i>R. cf. pseudopygmaeus</i> Hand.-Mazz.	Nepal; Hörandl & Emadzade 9689; WU	+	+	+
<i>R. psilostachys</i> Griseb.	cult. Lund BG; Johansson 219; LD	AY680106	AY954170	+
<i>R. pulchellus</i> C.A.Mey	Nepal; Hörandl & Emadzade 9679; WU	+	+	+
<i>R. punctatus</i> Jurtzev	Russia; Zimarskaya & al. s.n.; LE	FM242818	FM242754	+
<i>R. pygmaeus</i> Wahlenb.	Sweden; Larson & Granberg 9345; WU	AY954242	AY954122	+
<i>R. pyrenaicus</i> L.	Spain; Schneeweiss & al.; 6498 WU	AY680074	AY954225	GU258041
<i>R. radicans</i> C.A. Mey.	Mongolia; Schamsran 44272, HAL	FM242857	FM242793	+
<i>R. rarae</i> Exell	Malawi; Gehrke <i>et al.</i> BG-Af 304, ZH	EU288416	EU288389	+
<i>R. regelianus</i> Ovcz.	Pamir; Vasak s.n.; W	+	+	+
<i>R. repens</i> L.	Iran; Emadzade 107; WU	+	+	+
<i>R. reptans</i> L.	Switzerland; Willi br3; Z	AY680186	AY954205	+
<i>R. rufosepalus</i> Franch.	Pakistan; Millinger392897; LI	AY680047	AY954121	GU258042
<i>R. rumelicus</i> Griseb	Greece; Snogerup 5993b; LD	AY680104	AY954168	+
<i>R. sardous</i> Cr.	Sweden; Johansson s.n.; -	AY680122	AY954186	+
<i>R. sartorianus</i> Boiss. & Heldr.	cult. Copenhagen BG; Johansson 271; LD	AY680095	AY954148	+
<i>R. sceleratus</i> L.	Iran; Emadzade 112; WU	GU257971	GU257993	GU258043
<i>R. seguieri</i> ssp. <i>seguieri</i> Vill.	cult. Gothenburg BG; Johansson 226; LD	AY680079	AY954215	+
<i>R. septentrionalis</i> Poir.	USA; Raven <i>et al.</i> 27447; LE	FM242832	FM242768	+
<i>R. serbicus</i> Vis.	cult. Mühlhausen BG; Johansson 249; LD	AY680166	AY954196	+
<i>R. sericeus</i> Banks & Soland.	Iran; Emadzade 121; WU	+	+	+
<i>R. serpens</i> ssp. <i>nemorosus</i> (DC.) G. Lopez Gonzalez	Austria; Hörandl 9522; WU	AY954243	AY954184	+
<i>R. silerifolius</i> Lev.	Taiwan; Huang 1884; HAST	+	+	+
<i>R. sojakii</i> Iranshahr & Rech. f.	Iran; Emadzade 122; WU	+	+	+
<i>R. sphaerospermus</i> Boiss. & Blanche	Turkey; Dahlgren B87B; LD	AY680066	AY954132	GU258044
<i>R. spicatus</i> Desf.	cult. Wisley Bot. Garden, Johansson s.n. LD	AY954244	AY954158	+

<i>R. sprunerianus</i> Boiss.	Greece; Johansson 230; LD	AY680105	AY954169	+
<i>R. stagnalis</i> Hochst. ex A. Rich.	Ethiopia; Gehrke & al. BG-Af 228; ZH	EU288419	EU288392	+
<i>R. strigillosus</i> Boiss. & Hutt	Iran; Emadzade 117; WU	+	+	+
<i>R. submarginatus</i> Ovcz.	Russia, Altai; Pobedimova 52; LE	FM242841	FM242777	+
<i>R. taisanensis</i> Hayata	Taiwan; Yang & al. 7474; TNM	+	+	+
<i>R. tembensis</i> Hochst. ex A. Rich.	Ethiopia; Gehrke & al. BG-Af 210; ZH	EU288421	EU288393	+
<i>R. tenuirostris</i> J.Q.Fu	China; Podlech 55472; M	+	+	+
<i>R. ternei</i> Iranshahr & Rech. f.	Iran; Mozaffarian 54814; TARI	+	+	+
<i>R. thora</i> L.	cult. Lund BG; Johansson 223; LD	AY680188	AY954210	GU258045
<i>R. traunfellneri</i> Hoppe	Austria; Hörandl 2518; WU	AY954245	AY954222	+
<i>R. trichophyllus</i> Chaix	Greece; DahlgrenB23; LD	AY680067	AY954133	GU258046
<i>R. trilobus</i> Desf.	cult. Antwerpen BG; Johansson 217; LD.	AY680149	AY954176	+
<i>R. uncinatus</i> D. Don.	USA; Holmgren 5379; ZT	GU257972	GU257994	GU258047
<i>R. velutinus</i> Schur	cult. Rotterdam BG; Johansson 270; LD	AY680173	AY954198	+
<i>R. venetus</i> Huter ex Landolt	Italy; Gutermann & al. 35349; WU	AY680087	AY954144	+
<i>R. villarsii</i> DC.	Austria; Hörandl 664; WU	AY680099	AY954153	+
<i>R. volkensii</i> Engl.	Uganda; Gehrke & al. BG-Af 353; ZH	EU288424	EU288396	+
<i>Trautvetteria grandis</i> Honda	cult. California BG; Johansson 82.1322; -	AY680202	AF007945	GU258013

+ Sequences which will be submitted to GenBank for publishing.

### 2.3. Sequence alignment and phylogenetic analysis

The sequences were initially aligned using Clustal X (Thompson *et al.*, 1997). Subsequent corrections were carried out manually using BioEdit version 7.0.9.0 (Hall, 1999). Indels were treated as binary characters following the “simple indel coding method” (Simmons and Ochoterena, 2000) using the program SeqState version 1.36 (Müller, 2005). Nuclear and chloroplast sequences were analyzed separately and combined. A heuristic search for most parsimonious (MP) trees was performed with PAUP\* version 4.0b8 (Swofford, 2002). The analyses involved 1000 replicates with stepwise random taxon addition, tree bisection–reconnection (TBR) and branch swapping saving no more than 10 trees per replicate. All characters were equally weighted and treated as unordered (Fitch, 1971). Strict consensus trees were computed from all equally most parsimonious trees. Internal branch support was estimated using non-parametric bootstrapping (Felsenstein, 1985) with 1000 replicates and 10 addition sequences replicates.

Due to effect of modelling sequence evolution for different genes on tree topology accuracy (Sullivan and Swofford, 2001; Nylander *et al.*, 2004), a Bayesian inference approach was used to reconstruct phylogeny in addition to maximum parsimony. Different partitions of the data set, ITS, *matK*, *trnK*, and *psbJ-petA*, were tested using Mr Modeltest 2.2 (Nylander, 2004) separately to determine the sequence evolution model that best described the present data. A GTR+I+ $\Gamma$  substitution model was used for all partitions for final analysis using Mr.Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Four Markov chains were run simultaneously for 5,000,000 generations, and these were sampled every 1000 generations. Data from the first 1000 generations were discarded as the ‘burn-in’ period, after confirming

that likelihood values had stabilized prior to the 1000th generation. A majority rule consensus phylogeny and posterior probability (PP) of nodes were calculated from the remaining sample.

#### 2.4. Optimization of ancestral distributions

We used three methods, the parsimony-based method (DIVA v. 1.1, Ronquist, 1997), Mesquite v. 2.6 (Maddison and Maddison, 2009), and a maximum likelihood-based method (Lagrange v. 2.0.1, Ree and Smith, 2008) to infer vicariance and dispersal events. DIVA assumes that speciation is the result of vicariance, e.g. either a split of a wide distribution in two areas, or a speciation event within a single area, in which the two daughter species remain in their native area immediately after speciation (Ronquist, 1996, 1997). The program Lagrange (Ree and Smith, 2008) not only finds the most likely ancestral areas at a node and the split of the areas in the two descendant lineages, it also calculates the probabilities of these most-likely areas at each node (Ree and Smith, 2008). Lagrange was employed here with a simple model of one rate of dispersal and extinction constant over time and among lineages. We further reconstruct ancestral states based on parsimony using Mesquite (Maddison and Maddison, 2009). We used an ultrametric tree using the Bayesian analysis program BEAST v1.4.5 (Drummond and Rambaut, 2007) for all biogeographical analyses.

Distribution data were compiled from the literature (e.g., Ovczinnikov, 1937; Meusel *et al.*, 1965; Lourteig, 1984; Riedl and Nasir, 1990; Iranshahr *et al.*, 1992; Tutin and Cook, 1993; Rau, 1993; Whittmore, 1997; Wencai and Gilbert, 2001) to assign species to the five major geographic areas: Eurasia, N. America, S. America, Africa, and Hawaii. Mediterranean species that extend their distribution along the coast of North Africa were coded as Eurasian only. The distribution of each species of *Ranunculus* included in this analysis is shown in Figs. 2a, b, and Appendix 1.

For the study of details of historical biogeography in the Eurasian clades (“Tethyan clade”) as a model of a restricted area, Eurasia was subdivided into an eastern and a western Mediterranean area (including North Africa), Circumboreal, Irano-Turanian (excluding C. Asian high mountains), Central Asia, Eastern Asia, and the Himalaya-India region. Because the distribution matrix containing 48 taxa and 6 areas was too large to be read by DIVA, the optimization was performed in two steps of the phylogenetic tree (Fig. 2b, upper part and lower part). First, analysis of section A was processed alone and reduced to a single branch with its optimized areas. Analysis of section B included this single branch (Ronquist, 1996).

### 3. Results

#### 3.1. Phylogenetic analyses

The MP analyses of all 205 species based on ITS, revealed 70 most parsimonious trees with CI = 0.3609 and RI = 0.8241, while chloroplast markers only (*matK/trnK*, *psbj-petA*) revealed 60 most parsimonious trees with CI = 0.4772 and RI = 0.8503. The MP analysis of combined data resulted in 1120 most parsimonious trees with CI = 0.4152 and RI = 0.8260. The ITS analysis did not resolve well relationships within *Ranunculus*, showing a basal polytomy (not shown). The strict consensus tree of the chloroplast DNA overall showed better statistical support. As in previous studies (Paun *et al.*, 2005, Gehrke and Linder, 2009; Hoffmann *et al.*, 2010), the main clades were retained with both datasets (not shown). Parsimony analysis of the combined data set revealed a better resolution and higher statistical support than the results of either data set alone (Figs. 1a and b).

The topology provided by maximum parsimony (MP) of the combined data displays nine well supported clades (Figs. 1a and b; clades I-IX) which represent widespread ecological groups as in previous studies (Johansson, 1998; Hörandl *et al.*, 2005; Paun *et al.*, 2005; Lehnebach, 2008; Gehrke and Linder, 2009; Hoffmann *et al.*, 2010). The monophyly of these clades is well supported; however, their relationship between each other yields only weak support. Bayesian inference (BI) reveals the same clades (I-IX) as the MP tree topology, with overall higher resolution between clades and high posterior probabilities (PP) for clades (Fig. 1a). Both analyses confirm the separation of allied genera from *Ranunculus* s.str. (Fig. 1b). The core *Ranunculus* clade shows a gross subdivision into a group of clades, tending to colder and more humide areas, or aquatic habitats (Fig. 1b, clade A, I-IV), while a big clade with 94% BS and 100% PP units most species from the (boreal) temperate to meridional (tropical) zones, tending to mesic and dry habitats (Fig. 1a, clade B, V-IX).

Clades I-IV (Fig. 1b, clade A, alpine-arctic-wetland clades) mainly comprise species of high altitudes, latitudes, and wetlands. These clades show in general a low resolution which may be due to reticulate evolution, polyploidy and/or rapid speciation (see Hörandl *et al.*, 2005). The flammula clade (Fig. 1a, clade V) has a basal position in clade B with 100% BS and PP. The Tethyan, arvensis, acris, polyanthemus clades formed a tetrachotomy (Huber, 2003) in either MP or BI analyses with 100% BS and PP (Fig. 1a), although each of these clades is well supported. The arvensis clade and the Tethyan clade (Fig. 1a, clades VI, IX) consist of Eurasian species, and both are well supported in both MP and BI analyses (100% BS, 100% PP). The two remaining well supported clades (acris, polyanthemus clades; 100,

91% BS, respectively, and 100% PP for both) comprise species from all continents: Eurasia, North America, South America, Africa, and Oceania.

### 3.2 Biogeographical analyses

For biogeographical investigations we chose the meridional-temperate clades V-IX, (Fig. 1a, Tethyan, acris, arvensis, polyanthemos, and flammula clades and analyzed each of them separately. Ancestral area reconstructions from DIVA and Lagrange analyses resulted in more or less similar distribution ranges for all nodes (Appendix 1). In general, DIVA mostly reconstructs few ancestral areas that include all areas which are occupied by extant lineages; however, Lagrange shows a few ancestral areas with combinations of limited number of areas.

The most recent common ancestor (MRCA) of the flammula clade (Fig. 1a, clade V) occupied according to DIVA four areas: Eurasia, Africa, North America, and South America (Fig. 2a, node 1). *Ranunculus meyeri* from Africa and *R. hydrophilus* from South America are sister in this clade (Fig. 2a, node 2, 53% BS, 100% PP). DIVA reconstructed the MRCA of these two species in Africa and South America, although, Lagrange and Mesquite revealed it in Africa, and equivocal, respectively. South American species (*R. flagelliformis*) and Eurasian species (*R. ophioglossifolius*) make a clade with 100% PP (Fig. 2a, node 3). Lagrange and Mesquite suggested that the current distribution of these two species is due to dispersal from Eurasia to South America (with 0.9166 Rel. Prob., in Lagrange). DIVA, however, suggested a vicariance event for this node. The African species *R. volkensii* shows close relationships to eastern Asian and Indian species (Fig. 2a, node 4, 69% BS, 100% PP). Lagrange and Mesquite revealed that Eurasia was occupied by the MRCA of this clade (with 0.7199 Rel. Prob., in Lagrange). DIVA however, placed the MRCA within Eurasia and Africa and suggested vicariance for the current distribution of these species.

The acris clade (Fig. 2a, clade VI) comprises Eurasian, North American, and African species. DIVA, Lagrange and Mesquite analyses reconstructed Eurasia as the ancestral area of this clade (Fig. 2a, node 5). Some North American species (*Ranunculus occidentalis* and *R. uncinatus*) are sister to a Eurasian clade (*R. baldschuanicus* and *R. cassius*), which is sister to an African clade (*R. stagnalis* and *R. tembensis*), but without high support (Fig. 2a, node 6). Lagrange and Mesquite reveals the MRCA of this clade in Eurasia (with 0.7918 Rel. Prob., in Lagrange). DIVA reconstructs the distribution of the MRCA in all three areas or in Eurasia and Africa (Fig. 2a, node 6).

The polyanthemos clade (Fig. 2a, clade VIII) is widespread. DIVA reconstructed the ancestral area of this clade in Eurasia, North America, Africa, and South America or in

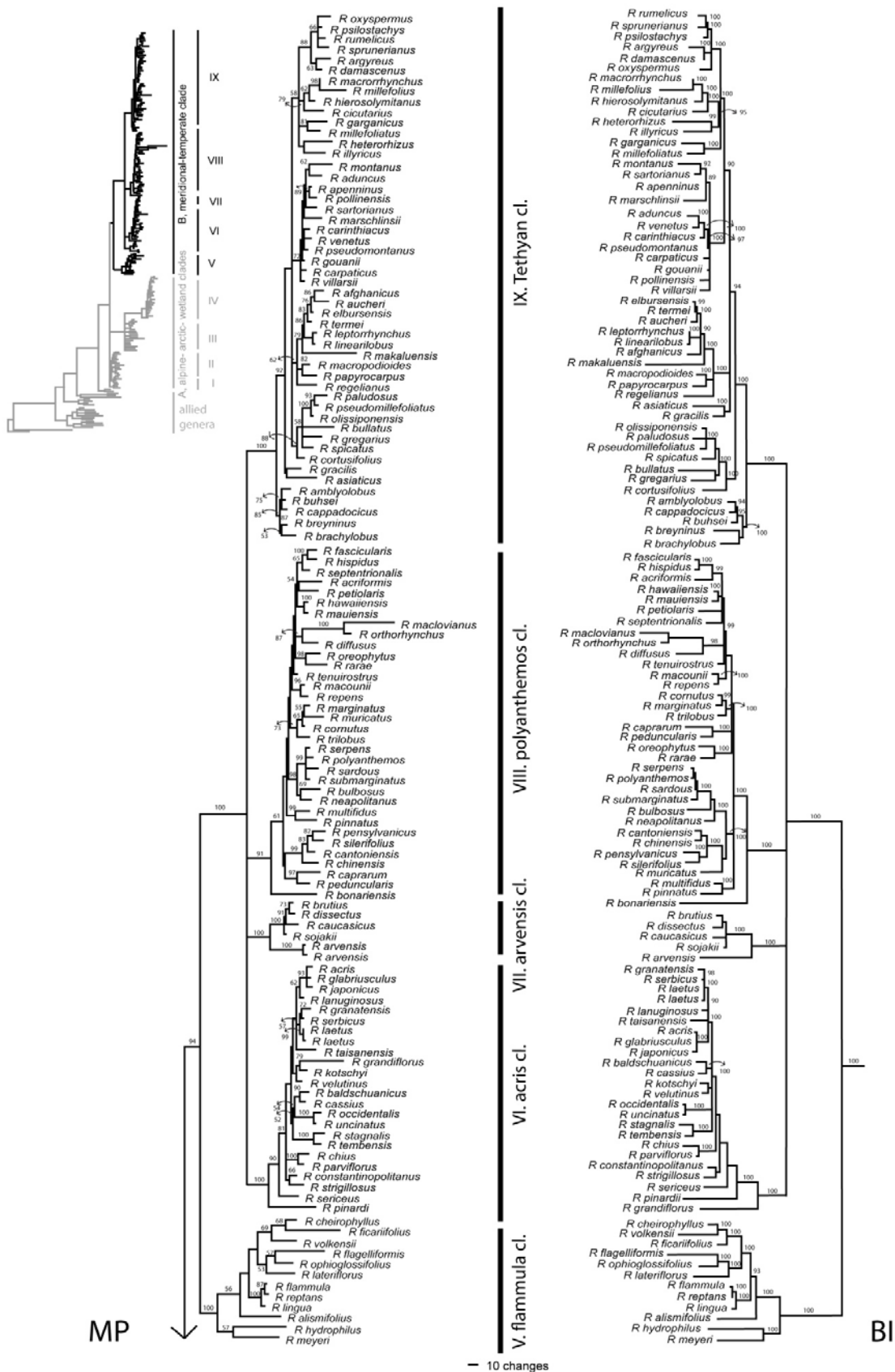
Eurasia and South America (Fig. 2a, node 7). Biogeographical analyses revealed multiple colonizations of all regions. There is evidence of disjunctions of North American and Eastern Asian species (*R. pensylvanicus*, *R. silerifolius*, Fig. 2a, node 8, 82% BS and 100% PP) and of North American and Eurasia (*R. macounii* and *R. repens* Fig. 2a, node 9, 96% BS and 100% PP) in this clade. In both cases, DIVA reconstructs the MRCA of these species in Eurasia and North America but Lagrange and Mesquite revealed it in Eurasia (with 0.6628 and 0.6712 Rel. Prob. respectively, in Lagrange). Africa has been colonized at least two times independently in this clade which confirmed previous studies (Gehrke and Linder, 2009). The colonization of the Hawaiian archipelago occurred from North America (Fig. 2a, node 11).

The *arvensis* clade (Fig. 2b, clade VII) includes five species. The Eurasian widespread *R. arvensis* is sister to the remaining species which have restricted distribution areas in the western Irano-Turanian and eastern Mediterranean regions. DIVA and Lagrange analyses showed that the MRCA of this clade had a widespread distribution in Eurasia identical to the area currently inhabited by *R. arvensis* (Fig. 2b, node 12), while the Mesquite analyses reconstructed the distribution of the MRCA in the Irano-Turanian region.

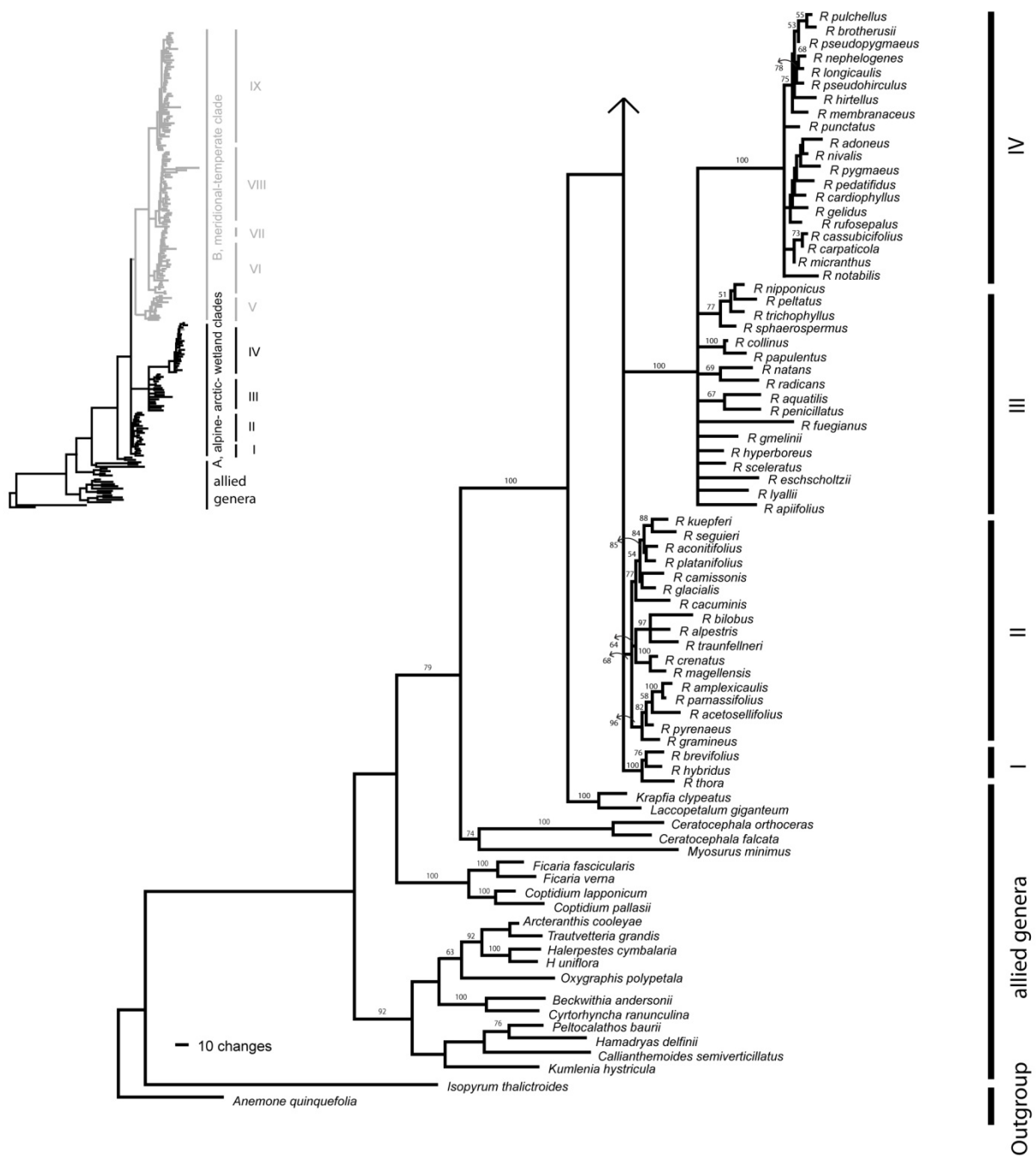
None of the biogeographical analyses revealed the ancestral area of the Tethyan clade (Fig. 2b, clade IX) unambiguously. The biogeographical history in the Tethyan clade shows several colonization events of the Irano-Turanian, the western and the eastern Mediterranean regions. Eastern Mediterranean species mostly show close relationships to Irano-Turanian species, rather than to western Mediterranean and Makaronesian species. Circumboreal species and high mountain European species are nested within the Tethyan clade, indicating a migration of buttercups from lower to higher altitudes/latitudes (Fig. 2b, nodes 20, 21).

**Table 2** Age estimates for nodes identified in Figs. 2a and b.

Node	Mean (Myr)	
	Emadzade & Hörandl (submitted)	Hoffmann & al. (2010)
<b>2</b>	< 5	< 4
<b>4</b>	< 2.5	< 3.5
<b>8</b>	< 1.2	< 6.5
<b>9</b>	< 3.3	ca. 3.0
<b>10</b>	< 2.5	< 4
<b>13</b>	ca. 12.8	< 11
<b>15</b>	ca. 6.99	< 7.0
<b>16</b>	< 7.0	< 7.0
<b>17</b>	< 2.5	-
<b>20</b>	< 3.6	< 5.0



**Figure 1a** Phylogenetic relationships of *Ranunculus* species and allied genera of the combined plastid and ITS dataset based on (a) Parsimony analyses, and (b) Bayesian inference. Bootstrap value  $\geq 50$  and posterior probability values  $\geq 90$  are indicated above branches. Tree overview is presented in the upper left-corner.



**Figure 1b** Phylogenetic relationships of *Ranunculus* species and allied genera of the combined plastid and ITS dataset based on parsimony analyses. Bootstrap value  $\geq 50$  is indicated above branches. Tree overview is presented in the upper left-corner.

## 4. Discussion

### 4.1. *Spatial-temporal development of the Temperate-Meridional clade buttercups*

The phylogenetic analysis of temperate–meridional groups revealed that closely related species occupy very distinct ranges with intercontinental disjunctions in clades V, VI, and VIII (Fig. 2a), as it was observed in related genera as well (Emadzade and Hörandl, submitted). However, the processes that have caused the frequent disjunctions between continents can be only understood in a global spatial-temporal framework. Previous age estimates showed that the stem and crown age group of the temperate-meridional clade (B) date back to the early and mid Miocene, respectively (Paun *et al.*, 2005; Hoffmann *et al.*, 2010; Emadzade and Hörandl, submitted; Table 2). Together with this age information, we attempt a reconstruction of the main biogeographical routes within and between continents. The Eurasian *arvensis* and the Tethyan clades show distinct geographical groups, suggesting regional radiations after geographical separation. We use the well-sampled Tethyan clade for a reconstruction of migration and diversification processes that might have occurred in other continents as well.

### 4.2. *Inter and intracontinental disjunctions*

#### 4.2.1. *North American-Eurasian disjunctions*

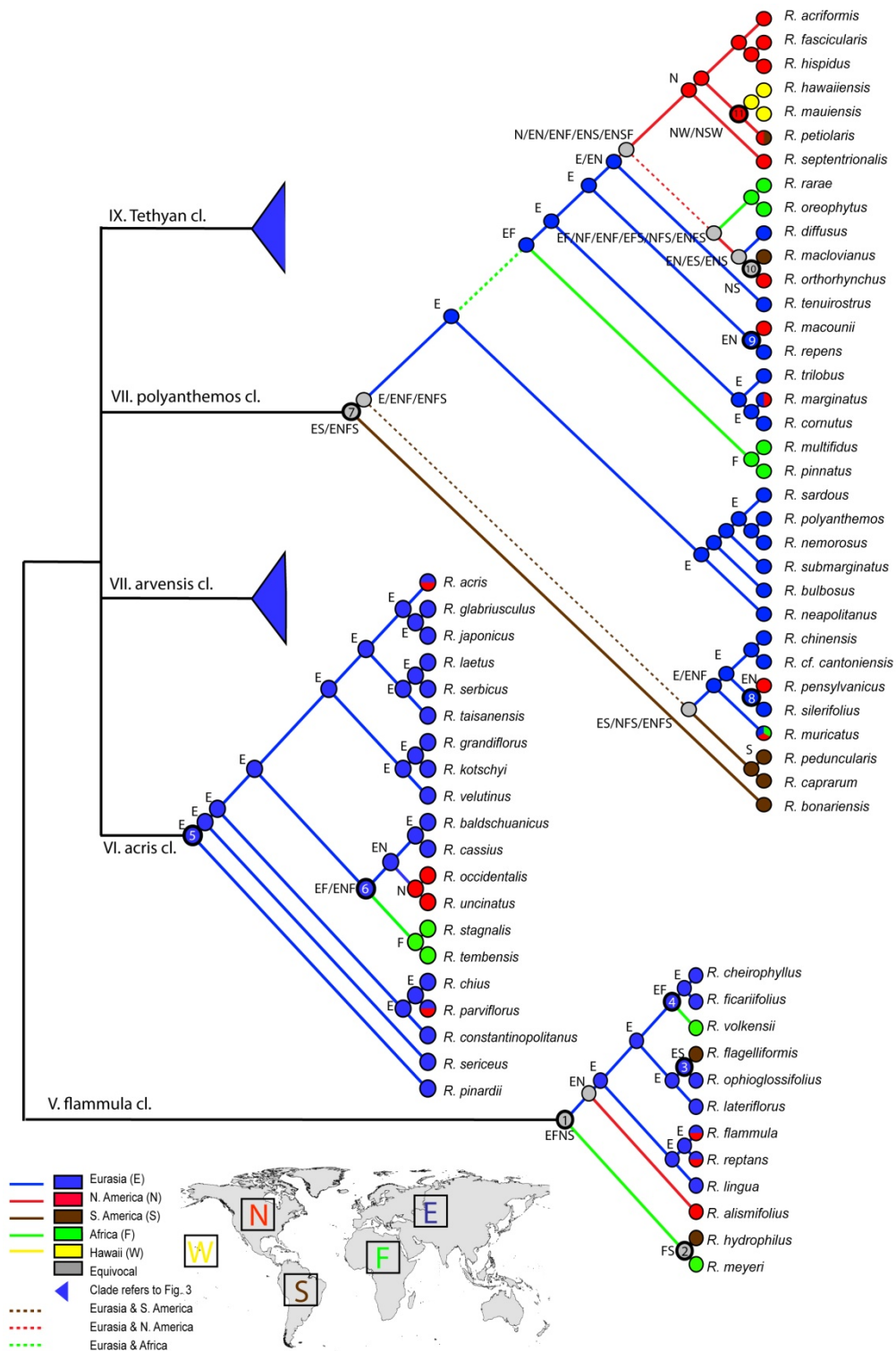
At least one disjunction event between East Asia (*R. silerifolius*) and North America (*R. pensylvanicus*) has happened in the Pleistocene (Table 2 and Fig. 2a, node 8, 82% BS, 100% PP), perhaps as a result of migrations from East Asia to North America (suggested by Mesquite and Lagrange; only DIVA suggested a vicariance event). This migration could have happened across the Bering land Bridge (BLB) or via long-distance dispersal (LDD) through the Pacific Ocean about 1-2 million years ago (Ma) (Fig. 3, arrow 7). The BLB has been thought to be a region of intercontinental exchange, and was believed to be available through most of the Cenozoic (Tiffney and Manchester, 2001). There is evidence that Beringia remained ice-free during the full glacial events of the Pleistocene and ruled as a refugial area (Hulten, 1937; Hopkins, 1959; Yurtsev, 1974, Cook *et al.*, 2005).

A connection of Eastern Asian and North America via Oceania and Australia is another reasonable hypothesis. Previous phylogenetic studies placed *R. pensylvanicus* as sister to a big clade comprising species from the Malesian Mountains and from Australia (Hörandl *et al.*, 2005; Lehnebach, 2008). However, since these studies did not include Eastern Asian species and were based on ITS sequence data only, they did not provide a robust phylogenetic

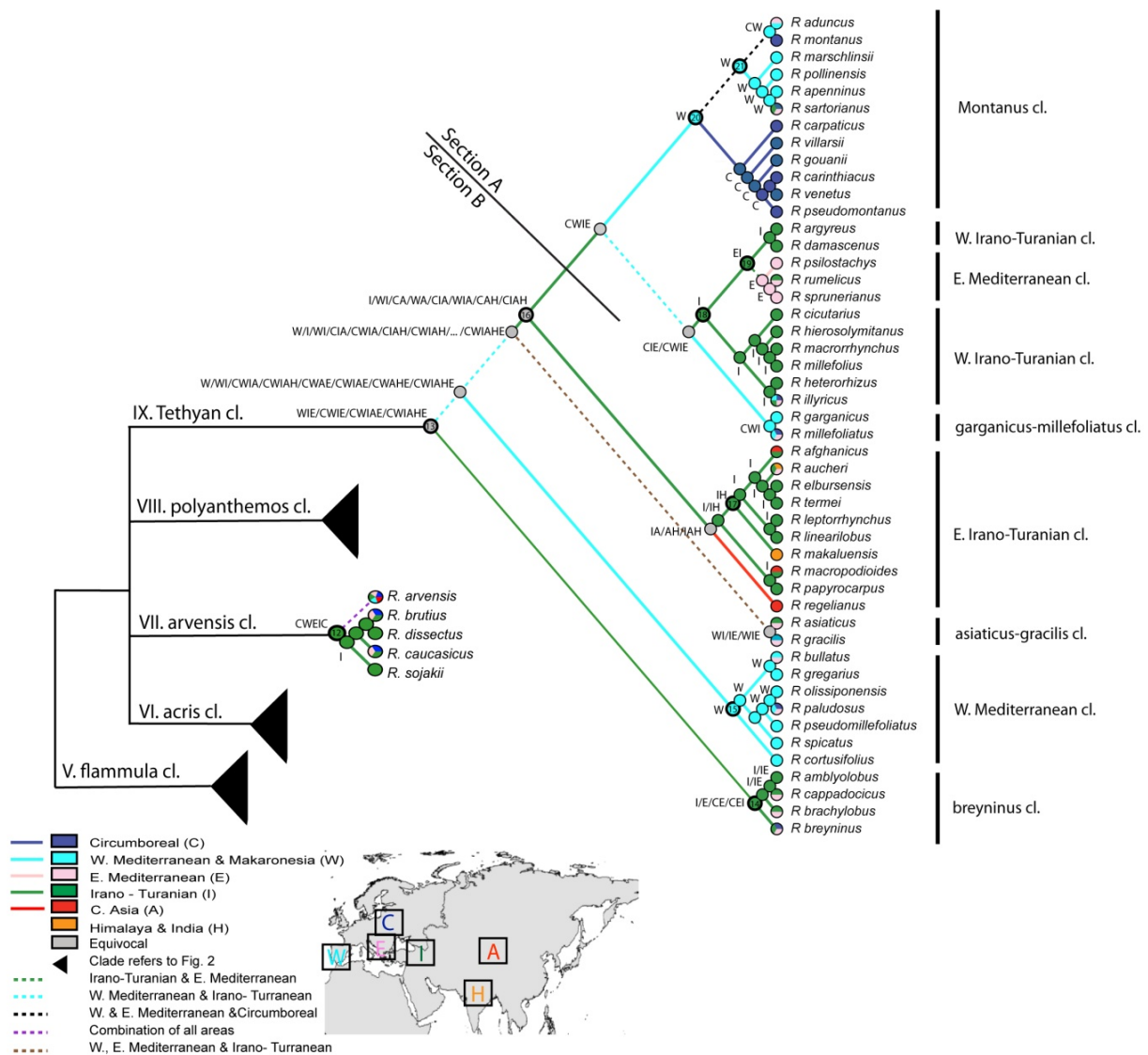
framework for a biogeographical hypothesis. Further studies are needed before a final conclusion can be drawn on this intercontinental disjunction.

The presence of other highly supported disjunctions (96%, 100% PP) in the polyanthemoid clade (Fig. 2a, node 9; *R. macounii*/*R. repens*) supports an exchange between North America and Eurasia. There are three possibilities to explain this distribution pattern: first, one could assume the presence of a widespread common ancestor in both areas split up by vicariance after the break-up of the North Atlantic Land Bridge (NALB) or Beringia Land Bridge (BLB), followed by allopatric speciation, as suggested by DIVA. Second, dispersal could have happened from Eurasia to North America aided by a number of stepping stones; third, a single long-distance dispersal event could explain the observed pattern of distribution. Both Lagrange and Mesquite support a dispersal hypothesis. A vicariance scenario has been observed by a number of studies that have investigated the southwest North American-Mediterranean disjunctions (Fritsch, 1996, 2001; Liston, 1997; Hileman *et al.*, 2001; Davis *et al.*, 2002). The migration of plants by hopping across the island chains is considered possible mainly in the Miocene (Wen, 1999; Tiffney, 2000; Manos and Donoghue, 2001). Because of the age of this split (Pleistocene, Table 2, node 9), a migration via the BLB is most likely. This pattern has also been reported in related genera of Ranunculaceae (Emadzade and Hörandl, submitted) as well as in other families (Blattner, 2005; Xiang *et al.*, 2000). However, transoceanic long-distance dispersal through the Pacific Ocean or the Atlantic Ocean cannot be ruled out. Long-distance dispersal through the Atlantic Ocean has been recorded in other genera (Fig. 3, arrow 8; Fritsch, 1996, 2001; Coleman *et al.*, 2001, 2003; Wen and Ickert-Bond, 2009).

Our data show that the Hawaiian Islands were colonized from North America (Fig. 2a, node 11) via long-distance dispersal, across the 3,900 km oceanic barrier (Fig. 3, arrow 11). On the basis of comparative floristic studies, most natural introductions of Hawaiian flowering plants were probably from Southeastern Asian source areas (Fosberg, 1948). Directionality of prevailing air currents, climatic similarities between the Hawaiian archipelago and Asian tropical areas are some arguments that support this idea. In contrast, about 20% of ancestral Hawaiian plant colonists are thought to have dispersed from the Americas, despite unfavorable prevailing winds and water currents (Fosberg, 1948, Geiger *et al.*, 2007; Harbaugh *et al.*, 2009). A close relationship to the Southern Pacific species *R. caprarum*, endemic to the Juan Fernandez archipelago, as hypothesized by Skottsberg (1922), is not supported by our data, because this species is sister to South American taxa (Fig. 2a).



**Figure 2a** Biogeographical optimization performed with the software DIVA, Lagrange, and Mesquite of meridional-temperate clade of *Ranunculus*: polyanthemos clade; acris clade; flammula clade. This tree is based on the combined plastid and ITS dataset. Relevant nodes are numbered (in circles). Most recent common ancestors reconstructed by DIVA are indicated on each node. Different lines show the highest probability migration routes suggested by Lagrange. Coloring shows ancestral area reconstruction under parsimony in Mesquite. Nodes of interest for this study are indicated by bold margin. Coded as stated in the figure: N, N. America; S, S. America; E, Eurasia; F, Africa; H, Hawaii.



**Figure 2b** Biogeographical optimization performed with the software DIVA, Lagrange, and Mesquite of meridional-temperate clade of *Ranunculus*: Tethyan clade; arvensis clade. This tree is based on the combined plastid and ITS dataset. Relevant nodes are numbered and indicated by bold margin (in circles). Most recent common ancestors reconstructed by DIVA are indicated on each node. Different lines show the highest probability migration routes suggested by Lagrange. Coloring shows ancestral area reconstruction under parsimony in Mesquite. Sections A and B refer to splitting of the tree for DIVA analysis (see materials and methods). Coded as stated in the figure: C, Circumboreal; W, W. Mediterranean and Makaronesia; E, E. Mediterranean; I, Irano-Turanian; A, C. Asia; H, Himalaya and India.

#### 4.2.2. South American-North American disjunctions

The South American species *R. maclovianus* is sister to the North American *R. orthorhynchus* with high bootstrap support in the polyanthemoid clade (Fig. 2a, node 10, 100% BS and PP). Based on our biogeographical analyses and previous age estimates, migration from North America to South America has probably happened in the Pleistocene (Table 2, node 10). Because the position of North and South America has not changed so much since the Cretaceous (Scotese, 2001), flora and fauna could exchange between North and South America through the Isthmus of Panama. On the other hand, disjunctions of plants between the west coast of North America and western South America have been reported several times (Carlquist, 1983; Vargas *et al.*, 1998). The extant widespread distribution of *R. orthorhynchus*, extending to western North America, makes coastal migration of the MRCA by birds more likely (Fig. 3, arrow 10; Wen and Ickert-Bond, 2009).

#### 4.2.3. South American-African disjunction

Our data suggest one disjunction event between South America (*R. hydrophilus*) and Africa (*R. meyeri*) in the Pleistocene (Table 2, Fig. 2a, node 2). Although DIVA suggested a vicariance event between South America and Africa, the corresponding age of this clade (Table 2, node 2), infers transoceanic dispersal between these two areas. A vicariance event due to the Gondwanaland breakup would be 130-100 Myr old (Lomolino *et al.*, 2006). *Ranunculus hydrophilus* and most other species in this clade occur in wetlands, where birds can be effective for dispersal. However, Lagrange showed dispersal from Africa to South America but the relative probability of this split is too low to be reliable (Fig. 3, arrow 2). Previous studies showed that LDD from South America to South Africa happened in monotypic genera of Ranunculeae (Emadzade *et al.*, submitted), and in other families (Givnish *et al.*, 2004, Schaefer *et al.*, 2009).

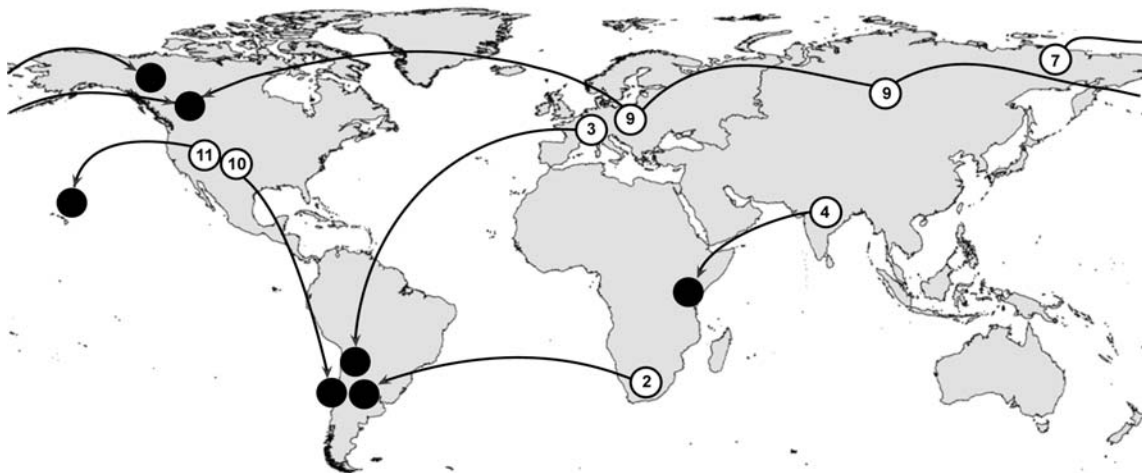
The African species are restricted to tropical alpine or high mountain habitats (Gehrke and Linder, 2009), and appear in three of the five studied clades (Fig. 2a). There are at least five colonization events of the African high mountains by *Ranunculus* as suggested by previous studies (Gehrke and Linder, 2009). Due to addition of more samples from all continents to the data set, our results did not confirm previous results that the African species are nested only within Northern Hemisphere clades (Gehrke and Linder, 2009). Our data show multiple colonizations of the tropical zones from temperate zones of the Southern and the Northern hemisphere.

#### 4.2.4. African-Asian disjunctions

A close relationship of African species (*Ranunculus volkensii*) to Asian species (*R. ficariifolius*, distributed in Nepal, Bhutan Sikkim and Thailand) and *R. cheirophyllus* (distributed in eastern Asia), is supported well in MP and BI analysis (Fig. 2a, node 4, 59% BS, 100% PP). According to the current distribution of these taxa and result of DIVA, one can in the first glance imagine the tectonic separation of the Indian subcontinent from Gondwanaland (150 Ma, Raval and Veeraswamy, 2003). But the very young diversification and speciation in this clade (<2.5 Ma, Table 2, node 4) make this hypothesis completely unlikely, and the best explanation is LDD from Asia to Africa, as suggested by Lagrange and Mesquite (Fig. 3, arrow 4).

#### 4.2.6. Asian-South American disjunctions

Transoceanic dispersal from Eurasia to South America is inferred from the sister relationship of the Mediterranean *R. ophioglossifolius* and the South American *R. flagelliformis* in the flammula clade (Fig. 2a, node 3; Fig. 3, arrow 3). Both species occur in wetlands and could have been distributed by birds. Long-distance dispersal over the Atlantic Ocean has been suggested in allied genera and other families with similar distributions as well (e.g. Wendel and Albert, 1992; Coleman *et al.*, 2001, 2003; Tremetsberger *et al.*, 2005; Emadzade and Hörandl, submitted).



**Figure 3** Intercontinental biogeographical connections at the tips of the phylogeny of meridional-temperate clade of *Ranunculus*. Arrows show important disjunctions between continents. Numbers in circles referred to the nodes of the tree in Fig. 2a. Black circles represent terminal taxa.

#### 4.3. The migration patterns in the Tethyan clade

Eurasia has been colonized by *Ranunculus* and related genera multiple times during the Neogene (Paun *et al.*, 2005; Hoffmann *et al.*, 2010; Emadzade and Hörandl, submitted). Although Eurasian species dominate in two of five clades (Tethyan and arvensis clades), they are present in the other three clades as well. The Eurasian species showed interchange with all other continents through land bridges or transoceanic dispersal (Fig. 3). The Tethyan clade is one of the two clades comprising Eurasian species only (with a few species colonizing also the Mediterranean zone of North Africa, e.g., *R. bullatus*). We chose the Tethyan clade to reconstruct the main process in forming the modern distribution of descents and effective factors in intracontinental dispersal in greater detail.

The origin of the Tethyan clade dates back to the middle Miocene (Table 2, node 13). The late Miocene and the early Pliocene are one of the most interesting stages of the Mediterranean and Paratethyan history. Geographically, this period was characterized by closures of the Betic and Rifian corridors and isolation of the Mediterranean Sea from the Atlantic Ocean, leading to very thick evaporate deposits in the Mediterranean area, known as the “Messinian Salinity Crisis” (Hsü *et al.*, 1973, Hsü, 1977; Agusti *et al.*, 2006a, b; Fauquette *et al.*, 2006; Popov *et al.* 2006). The crisis suddenly ended by the “re-flooding” of the Mediterranean basin through the Strait of Gibraltar at the beginning of the Pliocene (Agusti *et al.*, 2006b).

Although none of the biogeographical analyses could clearly reconstruct the ancestral area of the Tethyan clade (Fig. 2b, node 13) and other basal nodes with high probability, but with careful examination in the results, one can hypothesize the Mediterranean and the Irano-Turanian region as ancestral areas of this clade. Based on biogeographical analyses, probably a vicariance event in the Middle Miocene (Table 2, nodes 13, 15) isolated the breyninus subclade (Fig. 2b, nodes 13, 15) from its ancestors. It is possible that this split corresponded to the fluctuation of branchings out of the Paratethys and the maximum areal extension of Neo-Paratethys (Fig. 4a; Olteanu and Jipa, 2006).

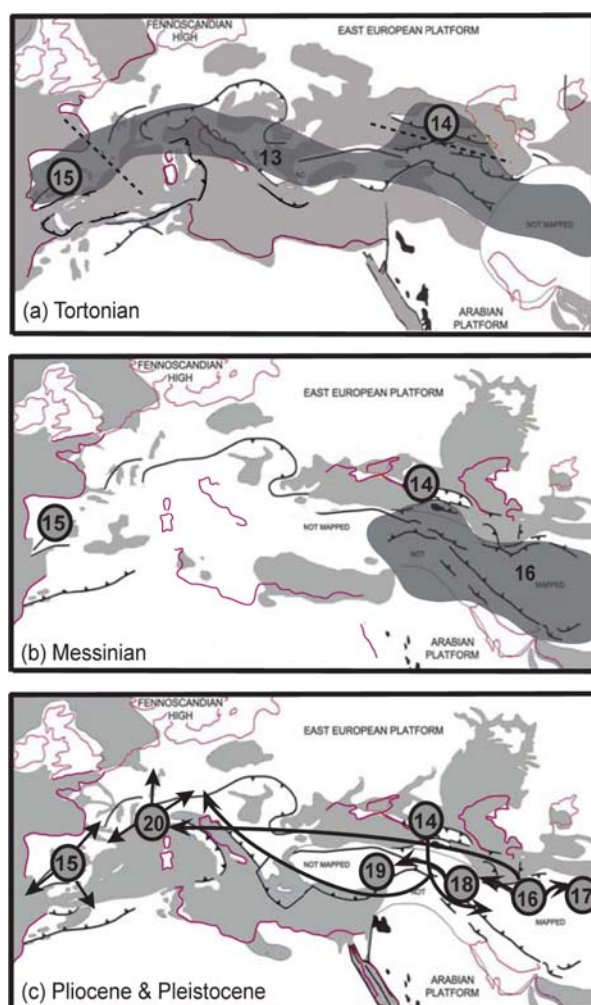
The breyninus subclade is sister to the other members of the Tethyan clade (100% BS, PP), and it is distributed in the high mountain ranges from the Caucasus region to the Alps with its centre of morphological diversity in the Caucasus region. The common ancestor of this subclade could have been isolated in the Greater Caucasus for about 5 Ma and then extended its distribution southwards to the Irano-Turanian region after retreat of the Paratethys. In the Pliocene and Pleistocene, *Ranunculus breyninus* colonized Turkey and the European mountains westwards to the Alps (Fig. 4b).

Limitation and migration of ancestors to the Irano-Turanian area took place in the late Miocene (Table 2 and Fig. 2b, nodes 16), presumably forced by the increasing aridity in the Mediterranean area during the Messinian Salinity Crisis (Fig. 4a and b). After this arid period the typical summer-dry and winter-wet Mediterranean climate stabilized c. 3 My ago, and an evergreen shrub vegetation established in southern Europe in the late Miocene (Suc, 1984; Willis and McElwaine, 2002; Thompson, 2005). In this period, buttercups re-colonized the Eastern Mediterranean area (E. Mediterranean clade, Fig. 2b), and diversified in this area similar to other Mediterranean radiations (e.g., in *Anthemis*, Lo Presti and Oberprieler, 2009). The diverse ecological conditions of the Mediterranean could have enhanced rapid adaptive radiations (e.g., Guzman *et al.*, 2009). The separation of the eastern Mediterranean and Irano-Turanian clades could be either explained by vicariance (suggested by DIVA) or by dispersal (suggested by Lagrange and Mesquite). Because of the geographical vicinity and the lack of strong geographical barriers, dispersal is more likely (Fig. 2b, node 19).

Our data indicate a closer relationship of the eastern Mediterranean region to the Irano-Turanian ancestors (Fig. 2b, node 19) than to the western Mediterranean buttercup flora. The isolation of areas during the Messinian salinity crisis (Fig. 4b) explains the differentiation between western and eastern part of the Mediterranean species as already observed in Paun *et al.* (2005). The western Mediterranean clade diversified probably after the onset of the Mediterranean climate in parallel to the eastern radiations, and even reached Makaronesia with one species, *R. cortusifolius*. All phylogenetic and biogeographical analyses support a close relationship of *R. cortusifolius* to Western Mediterranean species as observed by Paun *et al.* (2005), rather than to North African or eastern Mediterranean species (as suggested earlier by Bramwell and Richardson, 1973). Age estimates placed the origin of this species in the late Tertiary, when all of the Canary Islands were already formed (Carracedo, 1994).

The initial uplift of the European Alpine system about 10-2 Ma (Plaziat, 1981) provided opportunities for the evolution of alpine taxa. The subalpine-alpine montanus group (Fig. 2b, node 20) originated in the western to central Mediterranean, with at least one migration to the north/meridional to temperate and boreal zones in the Late Pliocene (Fig. 2b, node 20). As the diversification of this montanus clade began at the Plio-Pleistocene period (Table 2, node 20), its radiation could be related to the glaciation cycles of the Quaternary. Similar patterns were recorded in other mountain plant groups (Kadereit and Comes, 2005; Mraz *et al.*, 2007). In contrast to the observations in *Anthemis* by Lo Presti and Oberprieler (2009), buttercups show a progression of adaptations from summer-dry to montane humid climates. A multiple parallel colonization of high altitudes occurred in the eastern Irano-Turanian clade.

*Ranunculus makaluensis* originated from an ancestor in the eastern Irano-Turanian region about 3.8 Ma (Table 2 and Fig. 2b, node 17) and dispersed into the high altitudes of Eastern Himalaya, an area that is under the regime of the summer-monsoon. The species is a geographically isolated local endemic of the Makalu glacier region, growing in c. 4000-4500 m altitude (Kadota, 1991), and has no other Himalayan relative in this clade. Therefore, a long-distance dispersal event is likely. The other alpine Himalayan species are nested in clade IV, sister to North American, lowland European, and arctic species (Fig. 1b, clade IV). Therefore, the high altitudes of the Himalayas must have been colonized at least two times independently. In the E. Irano-Turanian clade, *R. elbursensis* is confined to the high alpine zones (Iranshahr *et al.*, 1992). Three species colonize the mountain steppes of C. Asia (*R. afghanicus*, *R. regelianus*, *R. macropodioides*), whereby the two former reach the subalpine and alpine zones (Ovcinnikov, 1937).



**Figure 4** Reconstruction of vicariance and dispersal events in Circum-Mediterranean *Ranunculus* based on biogeographical studies. Arrows and dashed lines depict predominant dispersal and vicariance events respectively. Numbers in circles referred to nodes of the tree in the Fig. 2b. The distribution of land mass and basins during different periods was based on maps given in Meulenkaamp and Sissingh (2003), Olteanu and Jipa (2006).

In summary, it is possible to distinguish three periods in the Tethyan clade: (1) colonization of the Mediterranean by a group of species in one (unknown) area in the middle Miocene (Fig. 4a); (2) a vicariance during the Messinian, isolating the ancestors of the *R. breyninus* clade in the Caucasus region and of the western Mediterranean clade (Fig. 4b); (3) range expansions and speciation in the Pliocene and Pleistocene (Fig. 4c), in the west extending to Makaronesia, in the east extending to the Eastern-Mediterranean, the Irano-Turanian regions and to Central Asia. Shifts to summer-wet climates in high mountain systems occurred three times independently: in the Alps by *R. breyninus* and the *montanus* clade, and in the Himalayas by *R. makaluensis*. In all regions, the colonization was followed by a rapid radiation and speciation.

#### 4.4. Capability of long-distance dispersal and rapid adaptation

Probably two main features could have made the genus cosmopolitan: successful dispersal over long distances, and establishment and survival in a wide range of habitats. Our data support multiple independent colonizations of different continents. These results reveal that long-distance dispersal may have played an important role for the worldwide distribution of *Ranunculus*. Three of five clades showed several intercontinental disjunctions within the Northern Hemispheric, Southern Hemispheric and between both hemispheric continents. The presence of endemic species of *Ranunculus* in some isolated oceanic islands, for instance on the Hawaii, Juan Fernandez, and Canarian Islands also suggests that LDD in this genus is possible. Long distance dispersal as an important factor of modern distributions of taxa is recorded in other genera of Ranunculaceae as well (Miikeda *et al.*, 2006; Ehrendorfer *et al.*, 2009; Emadzade and Hörandl, submitted). Molecular-based phylogenetic studies based on DNA sequences and estimates of divergence times of lineages supported the role of dispersal as a primary process shaping distribution patterns in both animals and plants (reviewed by de Queiroz, 2005).

However, achenes in buttercups do not have obvious adaptive morphological character to disperse by any vector, but Higgins *et al.* (2003) showed that the relationship between morphological features and long-distance dispersal is poor. Indeed, Green *et al.* (2008) showed collected achenes of *R. sceleratus* from faecal samples of *Anas gracilis* successfully germinated. Vagrant birds which are common bird throughout the world provide well-documented examples of vagrants involving distances large enough to explain long distance dispersal (e.g., Thorup, 1998). Especially the wide distribution of the flammula clade with

adapted species to wet habitats can be explained well with dispersal by birds. Local whirlwind or transoceanic whirlpool could carry simply the small and light achenes of *Ranunculi* as well. Indeed, transfer of achenes by wind (anemochory), bird (ornithochory), and water (hydrochory) has been documented in *Ranunculi* (Müller-Schneider, 1986).

Together with long-distance and transoceanic dispersal, *Ranunculus* could shift to quite different climatic regimes. In the Tethyan clade there was three times a shift from summer-dry climates (Irano-Turanian, Mediterranean) to summer-wet conditions (Alps, Himalayas). The endemic *R. makaluensis* originated from lowland Irano-Turanian species and reached the high alpine zone of the eastern Himalayas in Nepal, which is under the summer monsoon regime. The Alps were reached by the *R. montanus* group and by *R. breyninus*. Also in other continents, sister species may occur in contrasting climatic regimes, e.g. *R. petiolaris* (occurring in the continental climate of southern North America) and *R. hawaiiensis* and *R. mauiensis* (endemic to subtropical oceanic islands). It seems that *Ranunculus* has a high ability not only LDD to new areas but also to rapid adaptation to new habitats, whereby a tendency to moist habitats is predominant in all areas. In summer-dry climates, buttercups have developed special morphological adaptations like tuberous roots (Tamura, 1995; Paun *et al.*, 2005). In the subtropical and tropical zones, they shift to wetlands (e.g., *R. petiolaris*) or to high altitudes (e.g., the African or Himalayan species). In contrast, species colonizing the Arctic do not have any obvious novel morphological features, probably because of a pre-adaptation to wet habitats (Hoffmann *et al.*, 2010). The importance of physiological adaptations and chemical compounds needs to be studied. Establishment after long dispersal may be enhanced by different reproductive strategies, such as vegetative propagation, self-compatibility and agamospermy (Hörandl *et al.*, 2005; Hörandl, 2008). Moreover, buttercups have generalist flowers and therefore a broad spectrum of pollinators (Steinbach and Gottsberger, 1994), which may help for establishment in new environments.

However, the high potential for dispersal and colonization of new areas are not the only factors for the biogeographical history of *Ranunculus*. In the Tethyan clade we exemplify that geographical isolation is often followed by rapid speciation, probably due to strong adaptive radiations. Ecological differentiation into various micro-niches was recognized as a major factor for speciation in the *R. montanus* clade (Dickenmann, 1982). Polyploidy and hybridization can further contribute to sympatric speciation and diversification (Hörandl *et al.*, 2005). Previous studies based on ITS sequence data, but on a larger regional sampling indicated major radiations in New Zealand (Lockhart *et al.*, 2001; Lehnbech, 2008), Southern South America, Australia, and in the Malesian mountains (Hörandl *et al.*, 2005;

Lehnebach, 2008) similar to those in the Tethyan clade. The species-richness and the cosmopolitan distribution of *Ranunculus* are probably caused by the interplay of transoceanic plus intracontinental dispersal, and a potential for rapid adaptation and speciation.

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**Appendix S1** Ancestral area reconstructions for internal nodes of flammula, acris, arvensis, polyanthemus, and Tethyan clades, inferred using maximum parsimony (DIVA) and maximum likelihood (Lagrange).

## 1. Result of Lagrange analysis

Cladogram (branch lengths not to scale):

Ancestral range subdivision/inheritance scenarios ('splits') at internal nodes.

\* Split format: [left|right], where 'left' and 'right' are the ranges inherited by each descendant branch (on the printed tree, 'left' is the upper branch, and 'right' the lower branch).

\* Only splits within 2 log-likelihood units of the maximum for each node are shown. 'Rel.Prob' is the relative probability (fraction of the global likelihood) of a split.

### 1.1. flammula clade

#### Areas

**E: Eurasia**

**N: North America**

**S: South America**

**F: Africa**

**W: Hawaii**

```

-----+ [N] R_alismifolius
:
:
:-----+ [E] R_cheirophyllus
:
:-----N3+
:
:-----N5+-----+ [E] R_ficariifolius
-----N18+      :
:      :      :-----+ [F] R_volkensii
:      :      :-----N11+
:      :      :      :-----+ [S] R_flagelliformis
:      :      :      :-----N8+
:      :      :-----N10+-----+ [E] R_ophioglossifolius
:      :-----N17+      :
:      :      :-----+ [E] R_lateriflorus
N22+      :
:      :      :-----+ [EN] R_flammula
:      :      :-----N14+
:      :-----N16+-----+ [EN] R_reptans
:      :
:      :-----+ [E] R_lingua
:
:      :-----+ [S] R_hydrophilus
-----N21+
:-----+ [F] R_meyeri

```

Global ML at root node:

-lnL = 27.75

dispersal = 15.7

extinction = 16.47

**1.1.1. At node N22:**

split lnL Rel.Prob  
 [E|F] -29.92 0.1147  
 [E|S] -30.14 0.09183  
 [N|F] -30.19 0.08713  
 [F|F] -30.38 0.07194  
 [N|S] -30.41 0.06978  
 [F|FS] -30.7 0.0522  
 [EF|F] -30.78 0.04823  
 [E|EF] -30.93 0.04146  
 [E|E] -30.94 0.04113  
 [S|S] -30.96 0.04045  
 [S|FS] -31.06 0.03664  
 [E|ES] -31.12 0.03458  
 [ES|S] -31.19 0.03197  
 [N|FN] -31.21 0.03151  
 [N|N] -31.22 0.03125  
 [N|SN] -31.39 0.02628  
 [FN|F] -31.4 0.02612  
 [EN|E] -31.64 0.02045  
 [EN|N] -31.64 0.02045  
 [SN|S] -31.89 0.01595

**1.1.2. At node N18:**

split lnL Rel.Prob  
 [N|E] -29.03 0.278  
 [E|E] -29.49 0.1756

[N|EN] -29.69 0.144  
 [EN|E] -29.93 0.1134  
 [N|N] -30.59 0.05838  
 [F|E] -30.89 0.04354  
 [S|E] -30.99 0.03937

**1.1.3. At node N17:**

split lnL Rel.Prob  
 [E|E] -28.16 0.6673  
 [E|EN] -29.49 0.1751

**1.1.4. At node N11:**

split lnL Rel.Prob  
 [E|E] -28.08 0.7199  
 [EF|E] -29.29 0.2152

**1.1.5. At node N5:**

split lnL Rel.Prob  
 [E|F] -28.11 0.6973  
 [EF|F] -29.47 0.1792

**1.1.6. At node N3:**

split lnL Rel.Prob  
 [E|E] -27.94 0.8307

**1.1.7. At node N10:**

split lnL Rel.Prob

[E|E] -27.8 0.95

**1.1.8. At node N8:**

split lnL Rel.Prob  
 [E|E] -27.84 0.9166

**1.1.9. At node N16:**

split lnL Rel.Prob  
 [E|E] -28.34 0.5554  
 [EN|E] -28.65 0.4081

**1.1.10. At node N14:**

split lnL Rel.Prob  
 [N|EN] -29.29 0.214  
 [E|EN] -29.29 0.214  
 [EN|N] -29.44 0.1855  
 [EN|E] -29.44 0.1855  
 [E|E] -29.46 0.1817

**1.1.11. At node N21:**

split lnL Rel.Prob  
 [F|F] -28.78 0.3572  
 [S|S] -29.1 0.2599  
 [E|E] -30.38 0.07199  
 [N|N] -30.54 0.06143

## 1.2. acris clade

Areas

**E: Eurasia**

**N: North America**

**S: South America**

**F: Africa**

**W: Hawaii**

```

-----+ [EN] R_acris
----N2+
----N4+ -----+ [E] R_japonicus
:      :
:      -----+ [E] R_glabriusculus
---N10+
:      : -----+ [E] R_laetus
:      : ----N7+
:      ----N9+ -----+ [E] R_serbicus
---N16+      :
:      : -----+ [E] R_taisanensis
:      :
:      : -----+ [E] R_grandiflorus
:      : ----N13+
:      ----N15+ -----+ [E] R_velutinus
:      :
---N28+ -----+ [E] R_kotschyi
:      :
:      : -----+ [E] R_baldschuanicus
:      : ----N19+
:      :      : -----+ [E] R_cassius
:      : ----N23+
:      :      : -----+ [N] R_occidentalis
---N30+      :      : ----N22+
:      : ----N27+ -----+ [N] R_uncinatus
:      :      :
:      :      : -----+ [F] R_stagnalis
:      : ----N26+
---N34+      : -----+ [F] R_tembensis
:      :      :
:      : -----+ [E] R_constantinopolita.
:      :
---N36+      : -----+ [E] R_chius
:      : ----N33+
:      : -----+ [EN] R_parviflorus
N38+      :
:      -----+ [E] R_sericeus
:
-----+ [E] R_pinardii

```

Global ML at root node:

-lnL = 21.62

dispersal = 0.9858

extinction = 0.8136

**1.2.1. At node N38:**

split lnL Rel.Prob

[E|E] -21.79 0.8413

**1.2.2. At node N36:**

split lnL Rel.Prob

[E|E] -21.75 0.8735

**1.2.3. At node N34:**

split lnL Rel.Prob

[E|E] -21.74 0.8792

**1.2.4. At node N30:**

split lnL Rel.Prob

[E|E] -21.65 0.9656

**1.2.5. At node N28:**

split lnL Rel.Prob

[E|E] -21.85 0.7918

[E|EF] -23.4 0.1687

**1.2.6. At node N16:**

split lnL Rel.Prob

[E|E] -21.62 0.9988

**1.2.7. At node N10:**

split lnL Rel.Prob

[E|E] -21.62 0.9953

**1.2.8. At node N4:**

split lnL Rel.Prob

[E|E] -21.73 0.895

**1.2.9. At node N2:**

split lnL Rel.Prob

[E|E] -21.76 0.8656

[EN|E] -23.65 0.131

**1.2.10. At node N9:**

split lnL Rel.Prob

[E|E] -21.62 0.9997

**1.2.11. At node N7:**

split lnL Rel.Prob

[E|E] -21.62 0.9998

**1.2.12. At node N15:**

split lnL Rel.Prob

[E|E] -21.62 0.995

**1.2.13. At node N13:**

split lnL Rel.Prob

[E|E] -21.62 0.9946

**1.2.14. At node N27:**

split lnL Rel.Prob

[E|F] -21.92 0.7358

[E|E] -23.44 0.1613

**1.2.15. At node N23:**

split lnL Rel.Prob

[E|E] -21.7 0.9199

**1.2.16. At node N19:**

split lnL Rel.Prob

[E|E] -21.62 0.9955

**1.2.17. At node N22:**

split lnL Rel.Prob

[N|N] -21.72 0.9015

**1.2.18. At node N26:**

split lnL Rel.Prob

[F|F] -21.96 0.7106

[EF|F] -23.3 0.1854

**1.2.19. At node N33:**

split lnL Rel.Prob

[E|EN] -22.18 0.5706

[E|E] -22.5 0.4129

### 1.3. arvensis clade

Areas

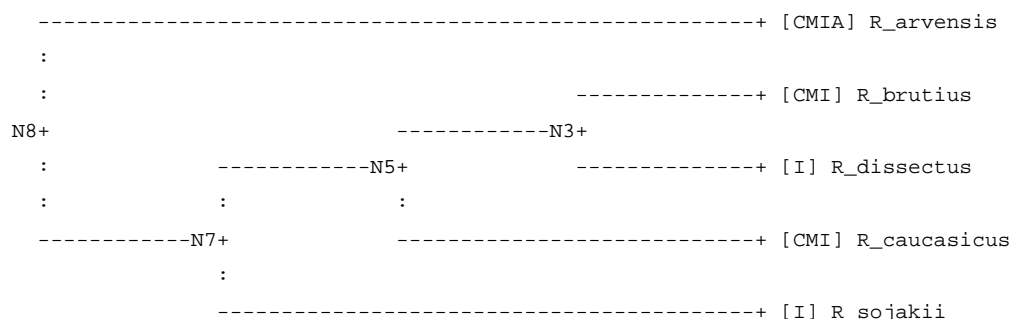
**C:** Circumboreal

**W:** West of Mediterranean

**E:** East of Mediterranean

**I: Irano-Turanian**  
**A: Central Asia**  
**H: Himalaya and India**

Cladogram (branch lengths not to scale):



Global ML at root node:

-lnL = 12.1  
dispersal = 96.07  
extinction = 50.84

#### 1.3.1. At node N8:

split	lnL	Rel.Prob
[CMIA I]	-13.52	0.2418
[CMIA M]	-13.65	0.2113
[CMIA C]	-13.65	0.2113
[CMIA A]	-13.68	0.2059

#### 1.3.2. At node N7:

split	lnL	Rel.Prob
[I I]	-13.39	0.2742
[CMIA I]	-13.86	0.1716

[CMI I]	-13.97	0.1541
[MI I]	-14.64	0.07868
[CI I]	-14.64	0.07868
[CMA I]	-15.03	0.05351
[CM I]	-15.29	0.04111

#### 1.3.3. At node N5:

split	lnL	Rel.Prob
[I I]	-13.45	0.2576
[I CMIA]	-14.93	0.05891
[I CMI]	-15.16	0.04694

[I CMA]	-15.25	0.04282
[I MI]	-15.44	0.03526
[I CI]	-15.44	0.03526

#### 1.3.4. At node N3:

split	lnL	Rel.Prob
[I I]	-12.72	0.535
[CMI I]	-14.34	0.1067
[MI I]	-14.53	0.08829
[CI I]	-14.53	0.08829

## 1.4. polyanthemom clade

**Areas**

**E: Eurasia**  
**N: North America**  
**S: South America**  
**F: Africa**  
**W: Hawaii**

```

-----+ [N] R_acrifomis
--N4+
:   :   ----+ [N] R_fascicularis
:   --N3+
-N10+   ----+ [N] R_hispidus
:   :
:   :   ----+ [W] R_hawaiiensis
:   :   --N7+
-N12+  --N9+   ----+ [W] R_mauiensis
:   :   :
:   :   -----+ [NS] R_petiolaris
:   :
-N22+  -----+ [N] R_septentrionalis
:   :
:   :   -----+ [F] R_rarae
:   :   ----N15+
:   :   :   -----+ [F] R_oreophytus
:   --N21+
-N24+   :   -----+ [E] R_diffusus
:   :   --N20+
:   :   :   ----+ [S] R_maclovianus
:   :   --N19+
-N28+   :   ----+ [N] R_orthorhynchus
:   :   :
:   :   -----+ [E] R_tenuirostrus
:   :
-N34+   :   -----+ [N] R_macoounii
:   :   ----N27+
:   :   -----+ [E] R_repens
:   :
:   :   -----+ [E] R_trilobus
-N38+  ----N33+
:   :   :   -----+ [EN] R_marginatus
:   :   ----N32+
:   :   -----+ [E] R_cornutus
:   :
:   :   -----+ [F] R_multifidus
:   ----N37+
:   :   -----+ [F] R_pinnatus
-N50+
:   :   -----+ [E] R_sardous
:   :   ----N43+
:   :   :   :   -----+ [E] R_polyanthemos
:   :   ----N45+   ----N42+
:   :   :   :   -----+ [E] R_serpens ssp. nemorosus
:   :   ---N47+   :
:   :   :   :   -----+ [E] R_submarginatus

```

```

      :   ---N49+      :
      :               :   -----+ [E] R_bulbosus
-N64+ :               :
      :               :   -----+ [E] R_neapolitanus
      :               :
      :               :   -----+ [E] R_chinensis
      :               :   -----N53+
      :               :   -----+ [E] R_cantoniensis
      :               :   -----N57+
      :               :   :   -----+ [N] R_pensylvanicus
N66+  :   -----N59+   -----N56+
      :   :   :   :   -----+ [E] R_silerifolius
      :   :   :   :
      :   -----N63+   -----+ [ENF] R_muricatus
      :               :
      :               :   -----+ [NS] R_peduncularis
      :   -----N62+
      :               :   -----+ [S] R_caprarum
      :
      -----+ [S] R_bonariensis

```

Global ML at root node:

-lnL = 71.23

dispersal = 6.258

extinction = 2.275

At node N66:

split	lnL	Rel.Prob	[EF E]	-72.22	0.3725
split	lnL	Rel.Prob	[E ES]	-72.97	0.1763
[ES S]	-73.06	0.1599	[ENF E]	-72.42	0.304
[ENS S]	-73.27	0.1304	[E E]	-72.66	0.2385
[S S]	-73.45	0.1092			
[EN S]	-73.51	0.1021			
[EFS S]	-73.67	0.0869			
[E S]	-73.86	0.07222			
[EF S]	-74.13	0.05497			
[NS S]	-74.17	0.05275			
[N S]	-74.31	0.046			
[NF S]	-74.34	0.0447			
[FS S]	-74.53	0.03691			

split	lnL	Rel.Prob
[E ES]	-72.97	0.1763
[E ENS]	-73.03	0.1649
[E EFS]	-73.94	0.06687
[E EN]	-74.26	0.04851
[E E]	-74.3	0.04651
[F ES]	-74.34	0.04442
[F EFS]	-74.51	0.03754
[N ENS]	-74.66	0.03255
[E NS]	-74.69	0.03136
[ENF N]	-74.74	0.02978
[N N]	-74.85	0.02682

At node N50:

split	lnL	Rel.Prob
-------	-----	----------

At node N64:

At node N38:

split	lnL	Rel.Prob
[E F]	-72	0.4657
[EN F]	-72.54	0.2695
[ENF F]	-73.27	0.1304
[EF F]	-73.92	0.06782

At node N34:

split	lnL	Rel.Prob
[E E]	-71.91	0.506
[EN E]	-72.72	0.2252

[ENF E] -73.37 0.1172	[N N] -71.23 0.9999	[E EN] -72.97 0.1759
At node N28:	At node N9:	At node N32:
split lnL Rel.Prob	split lnL Rel.Prob	split lnL Rel.Prob
[E E] -72 0.4616	[W N] -71.56 0.72	[E E] -71.61 0.6828
[EN E] -73.25 0.1322	[W NS] -73.1 0.1535	[EN E] -72.4 0.3107
[EN N] -73.25 0.1322		
[ENF N] -73.82 0.07474	At node N7:	At node N37:
[ENF E] -73.82 0.07474	split lnL Rel.Prob	split lnL Rel.Prob
	[W W] -71.25 0.9786	[F F] -71.26 0.9759
At node N24:		
split lnL Rel.Prob	At node N21:	At node N49:
[EN E] -72.28 0.3511	split lnL Rel.Prob	split lnL Rel.Prob
[N E] -72.68 0.2348	[F EN] -71.88 0.5247	[E E] -71.25 0.9956
[E E] -73.03 0.1651	[F E] -73.44 0.1099	
[ENF E] -73.1 0.1535	[F N] -73.44 0.1096	At node N47:
[NF E] -73.96 0.06541		split lnL Rel.Prob
	At node N15:	[E E] -71.25 0.9908
	split lnL Rel.Prob	
At node N22:	[F F] -71.26 0.9759	At node N45:
split lnL Rel.Prob		split lnL Rel.Prob
[N EN] -72.23 0.3674	At node N20:	[E E] -71.45 0.9985
[N N] -72.65 0.241	split lnL Rel.Prob	
[N E] -73.22 0.1374	[E N] -72.15 0.3983	At node N43:
[N ENF] -73.45 0.109	[E NS] -72.94 0.1813	split lnL Rel.Prob
[N EF] -73.98 0.06397	[E EN] -73.7 0.08492	[E E] -71.26 0.9967
[N NF] -74.22 0.05033	[E E] -73.88 0.07051	
	[E NF] -73.91 0.06876	At node N42:
At node N12:		split lnL Rel.Prob
split lnL Rel.Prob	At node N19:	[E E] -71.23 0.9983
[N N] -71.24 0.9916	split lnL Rel.Prob	
	[S N] -71.89 0.515	At node N63:
At node N10:	[N N] -73.32 0.1244	split lnL Rel.Prob
split lnL Rel.Prob		[E S] -72.65 0.2419
[N N] -71.25 0.9834		[EN S] -73.08 0.1571
	At node N27:	[EF S] -73.39 0.1152
At node N4:	split lnL Rel.Prob	[N N] -73.4 0.1147
split lnL Rel.Prob	[N E] -71.25 0.9806	[E NS] -73.92 0.06779
[N N] -71.23 0.9992		[EN N] -74.06 0.05896
	At node N33:	[E E] -74.46 0.03955
At node N3:	split lnL Rel.Prob	[N NS] -74.61 0.03416
split lnL Rel.Prob	[E E] -71.43 0.8168	

At node N59:

```
split lnL Rel.Prob
[E|E] -72.25 0.361
[E|EN] -73.17 0.1432
[E|EF] -73.37 0.1172
[N|N] -73.57 0.09627
[E|ENF] -73.95 0.06583
[EN|N] -74.01 0.06224
[EN|E] -74.01 0.06224
```

At node N57:

```
split lnL Rel.Prob
[E|E] -71.64 0.6628
[E|EN] -72.37 0.3201
```

At node N53:

```
split lnL Rel.Prob
[E|E] -71.23 0.999
```

At node N56:

```
split lnL Rel.Prob
[N|E] -71.27 0.9573
```

At node N62:

```
split lnL Rel.Prob
[S|S] -71.88 0.5251
[NS|S] -72.18 0.3861
```

## 1.5.Tethyan clade

Areas

**C:** Circumboreal

**W:** West of Mediterranean

**E:** East of Mediterranean

**I:** Irano-Turanean

**A:** Central Asia

**H:** Himalaya and India

```

-----+ [CIE] R_breyninus
-----N6+
:      :      -----+ [IE] R_brachylobus
:      -----N5+
:      :      -----+ [IE] R_cappadocicus
:      -----N4+
:      -----+ [I] R_ambelyolobus
:
:      -----+ [W] R_gregarius
:      -----N9+
:      :      -----+ [WE] R_bullatus
:      :
:      ---N17+      -----+ [CWE] R_paludosus
N94+      :      :      ----N12+
:      :      :      ----N14+      -----+ [W] R_olissiponensis
:      :      :      :      :
:      ---N19+      ----N16+      -----+ [W] R_pseudomillefoliatus
:      :      :      :
:      :      :      -----+ [W] R_spicatus
```

```

:   :   :
:   :   -----+ [W] R_cortusifolius
:   :
:   :   -----+ [WE] R_gracilis
:   :   -----N22+
:   :   :   -----+ [IE] R_asiaticus
:   :   :
-N93+ :   -----+ [A] R_regelianus
      :   :   :
      :   :   :   -----+ [I] R_papyrocarpus
      :   :   -N41+   -----N26+
      :   :   :   :   :   -----+ [IA] R_macropoioides
      :   :   :   :   :
      :   :   :   -N40+   -----+ [H] R_makaluensis
      :   :   :   :   :
      :   :   :   :   :   -----+ [I] R_leptorrhynchus
      :   :   :   -N39+   -----N30+
-N92+ :   :   :   -----+ [I] R_linearilobus
      :   :   :   :
      :   :   :   -N38+   -----+ [I] R_elbursensis
      :   :   :   :   -N33+
      :   :   :   :   -N35+   -----+ [I] R_termei
      :   :   :   :   :   :
      :   :   :   -N37+   -----+ [IHE] R_aucheri
      :   :   :   :
      :   :   :   -----+ [IA] R_afghanicus
      :   :
      :   :   -----+ [I] R_heterorhizus
      :   :   -----N44+
      :   :   :   -----+ [CWIE] R_illyricus
      :   :   -N52+
-N91+ :   :   -----+ [I] R_cicutarius
      :   :   :
      :   :   -N51+   -----+ [I] R_millefolius
      :   :   :   -N48+
      :   :   :   -N50+   -----+ [I] R_macrorrhynchus
      :   :   -N62+   :
      :   :   :   -----+ [I] R_hierosolymitanus
      :   :   :
      :   :   :   -----+ [E] R_sprunerianus
      :   :   :   --N55+
      :   :   :   --N57+   -----+ [IE] R_rumelicus

```

```

:      :      :      :      :
:  -N66+  --N61+  -----+ [E] R_psilostachys
:      :      :      :
:      :      :      :      -----+ [I] R_damascenus
:      :      :      ----N60+
:      :      :      -----+ [I] R_argyreus
:      :      :
:      :      :      -----+ [CWE] R_millefoliatus
:      :  -----N65+
:      :      -----+ [W] R_garganicus
:      :
-N90+      -----+ [C] R_carinthiacus
:      -N69+
:      -N71+  -----+ [C] R_venetus
:      :      :
:      -N73+  -----+ [C] R_pseudomontanus
:      :      :
:      -N75+  -----+ [W] R_gouanii
:      :      :
:  -N77+  -----+ [C] R_villarsii
:      :      :
:      :  -----+ [C] R_carpaticus
-N89+
:      -----+ [C] R_montanus
:  -----N80+
:      :      -----+ [WE] R_aduncus
-N88+
:      -----+ [W] R_marschlinsii
--N87+
:      -----+ [W] R_pollinensis
--N86+
:      -----+ [CWE] R_sartorianus
--N85+
:      -----+ [W] R_apenninus

```

Global ML at root node:

-lnL = 123.7

dispersal = 100

extinction = 4.285e-009

**1.5. 1. At node N94:**

split lnL Rel.Prob  
 [I|WI] -125 0.249  
 [I|WIE] -125.5 0.1665  
 [E|WIE] -125.8 0.1218  
 [E|WI] -126.1 0.09105

**1.5. 2. At node N6:**

split lnL Rel.Prob  
 [I|I] -124.4 0.4668  
 [E|E] -125.1 0.2317

**1.5. 3. At node N5:**

split lnL Rel.Prob  
 [I|I] -124.4 0.4795  
 [E|E] -125.2 0.2212  
 [IE|I] -126 0.1003  
 [E|IE] -126.4 0.06616  
 [I|IE] -126.4 0.06616

**1.5. 4. At node N4:**

split lnL Rel.Prob  
 [IE|I] -123.9 0.7578  
 [I|I] -125.1 0.2389

**1.5. 5. At node N93:**

split lnL Rel.Prob  
 [W|WI] -124.8 0.3134  
 [W|WIE] -125.1 0.245  
 [W|IE] -126.5 0.05675  
 [W|W] -126.7 0.04929  
 [W|CWIE] -126.7 0.04665  
 [W|I] -126.7 0.04559

**1.5. 6. At node N19:**

split lnL Rel.Prob  
 [W|W] -123.7 0.9956

**1.5. 7. At node N17:**

split lnL Rel.Prob  
 [W|W] -123.7 0.9522

**1.5. 8. At node N9:**

split lnL Rel.Prob  
 [W|W] -123.7 0.939

**1.5. 9. At node N16:**

split lnL Rel.Prob  
 [W|W] -123.7 0.9718

**1.5. 10. At node N14:**

split lnL Rel.Prob  
 [W|W] -123.8 0.8837

**1.5. 11. At node N12:**

split lnL Rel.Prob  
 [W|W] -123.8 0.8778

**1.5. 12. At node N92:**

split lnL Rel.Prob  
 [WIE|I] -125.5 0.1661  
 [WI|I] -125.6 0.1444  
 [I|WI] -125.9 0.1012  
 [W|WI] -126.3 0.06898  
 [I|I] -126.7 0.0473  
 [IE|I] -126.8 0.04422  
 [W|W] -127 0.03646

**1.5. 13. At node N22:**

split lnL Rel.Prob  
 [WE|I] -124.2 0.5751  
 [W|I] -125 0.2679  
 [E|I] -125.5 0.157

**1.5. 14. At node N91:**

split lnL Rel.Prob  
 [I|I] -124.6 0.4047  
 [I|WI] -125.2 0.2124

**1.5. 15. At node N41:**

split lnL Rel.Prob  
 [A|IA] -124.6 0.3991

[A|IH] -125.5 0.165  
 [A|ICH] -125.5 0.1633  
 [A|I] -125.5 0.1512

**1.5. 16. At node N40:**

split lnL Rel.Prob  
 [IC|I] -124.8 0.3197  
 [I|IH] -125.4 0.173  
 [I|I] -125.7 0.1257  
 [IC|H] -125.9 0.109  
 [I|ICH] -126.4 0.06603

**1.5. 17. At node N26:**

split lnL Rel.Prob  
 [I|IC] -124.1 0.6311  
 [I|I] -124.7 0.3635

**1.5. 18. At node N39:**

split lnL Rel.Prob  
 [H|I] -124.7 0.3541  
 [H|IH] -124.9 0.2854  
 [H|H] -125.8 0.1164  
 [H|IC] -125.9 0.1064

**1.5. 19. At node N38:**

split lnL Rel.Prob  
 [I|I] -124.8 0.3236  
 [I|IH] -124.9 0.3023  
 [I|IHE] -125.6 0.1503  
 [I|IC] -125.6 0.1443

**1.5. 20. At node N30:**

split lnL Rel.Prob  
 [I|I] -123.7 1

**1.5. 21. At node N37:**

split lnL Rel.Prob  
 [I|I] -124.8 0.3231  
 [IH|I] -124.9 0.3004  
 [IHE|I] -125.6 0.1495  
 [I|IC] -125.6 0.143

<b>1.5. 22. At node N35:</b>	<b>1.5. 31. At node N48:</b>	
split lnL Rel.Prob	split lnL Rel.Prob	split lnL Rel.Prob
[I I] -124.6 0.3714	[I I] -123.7 1	[C C] -124.1 0.6205
[I IHE] -124.9 0.2898		[CW C] -124.6 0.3792
[I IH] -124.9 0.2804	<b>1.5. 32. At node N61:</b>	<b>1.5. 40. At node N73:</b>
[I IE] -126.6 0.05206	split lnL Rel.Prob	split lnL Rel.Prob
	[IE I] -124.1 0.6216	[C W] -123.7 1
	[I I] -125 0.2582	
<b>1.5. 23. At node N33:</b>	[E I] -125.8 0.1202	<b>1.5. 41. At node N71:</b>
split lnL Rel.Prob		split lnL Rel.Prob
[I I] -123.7 1	<b>1.5. 33. At node N57:</b>	[C C] -123.7 1
	split lnL Rel.Prob	
<b>1.5. 24. At node N90:</b>	[IE E] -123.8 0.8722	<b>1.5. 42. At node N69:</b>
split lnL Rel.Prob		split lnL Rel.Prob
[W W] -124.2 0.5677	<b>1.5. 34. At node N55:</b>	[C C] -123.7 1
	split lnL Rel.Prob	
<b>1.5. 25. At node N66:</b>	[E IE] -123.7 0.923	<b>1.5. 43. At node N88:</b>
split lnL Rel.Prob		split lnL Rel.Prob
[I W] -124.2 0.555	<b>1.5. 35. At node N60:</b>	[CWE W] -124.4 0.4858
	split lnL Rel.Prob	[CW W] -124.9 0.2845
<b>1.5. 26. At node N62:</b>	[I I] -123.7 1	[W W] -125.3 0.185
split lnL Rel.Prob		
[I I] -123.9 0.7584	<b>1.5. 36. At node N65:</b>	<b>1.5. 44. At node N80:</b>
	split lnL Rel.Prob	split lnL Rel.Prob
<b>1.5. 27. At node N52:</b>	[W W] -124.1 0.6468	[C WE] -124 0.7134
split lnL Rel.Prob	[WE W] -125.6 0.1367	[C W] -124.9 0.2784
[I I] -123.8 0.8538	[CW W] -125.8 0.1123	
		<b>1.5. 45. At node N87:</b>
<b>1.5. 28. At node N44:</b>	<b>1.5. 37. At node N89:</b>	split lnL Rel.Prob
split lnL Rel.Prob	split lnL Rel.Prob	[W W] -123.7 0.9938
[I I] -124.1 0.6672	[C CWE] -124.6 0.3783	
[I WI] -125.9 0.104	[CW W] -125.1 0.2455	<b>1.5. 46. At node N86:</b>
	[C CW] -125.1 0.2424	split lnL Rel.Prob
<b>1.5. 29. At node N51:</b>	[CW C] -126.6 0.05382	[W W] -123.7 0.9802
split lnL Rel.Prob		
[I I] -123.7 1	<b>1.5. 38. At node N77:</b>	<b>1.5. 47. At node N85:</b>
	split lnL Rel.Prob	split lnL Rel.Prob
<b>1.5. 30. At node N50:</b>	[C C] -124.1 0.6722	[W W] -123.8 0.9046
split lnL Rel.Prob	[CW C] -124.8 0.3277	
[I I] -123.7 1		
	<b>1.5. 39. At node N75:</b>	

## 2. Result of DIVA analysis

### 2.1. flammula clade

**Areas**

**E: Eurasia**

**N: North America**

**S: South America**

**F: Africa**

**W: Hawaii**

>optimize;

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 23 0%

up & final on node: 13

optimization successful - exact solution

settings: maxareas=4, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 4 dispersals

optimal distributions at each node:

node 13 (anc. of terminals r\_cheirophyllus-r\_ficariifolius): E

node 14 (anc. of terminals r\_cheirophyllus-r\_volkensii): EF

node 15 (anc. of terminals r\_flagelliformis-r\_ophioglossifol): ES

node 16 (anc. of terminals r\_flagelliformis-r\_lateriflorus): E

node 17 (anc. of terminals r\_cheirophyllus-r\_lateriflorus): E

node 18 (anc. of terminals r\_flammula-r\_reptans): E

node 19 (anc. of terminals r\_flammula-r\_lingua): E

node 20 (anc. of terminals r\_cheirophyllus-r\_lingua): E

node 21 (anc. of terminals r\_alismifolius-r\_lingua): EN

node 22 (anc. of terminals r\_hydrophilus-r\_meyeri): FS

node 23 (anc. of terminals r\_alismifolius-r\_meyeri): EFSN

### 2.2. acris clade

**Areas**

**E: Eurasia**

**N: North America**

**S: South America**

**F: Africa**

**W: Hawaii**

>optimize;

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 39 0%

up & final on node: 21

optimization successful - exact solution

settings: maxareas=3, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 4 dispersals

optimal distributions at each node:

node 21 (anc. of terminals r\_acris-r\_japonicus): A

node 22 (anc. of terminals r\_acris-r\_glabriuscu): A

node 23 (anc. of terminals r\_laetus-r\_serbicus): A

node 24 (anc. of terminals r\_laetus-r\_taisanensis): A

node 25 (anc. of terminals r\_acris-r\_taisanensis): A

node 26 (anc. of terminals r\_grandiflorus-r\_velutinus): A

node 27 (anc. of terminals r\_grandiflorus-r\_kotschy): A

node 28 (anc. of terminals r\_acris-r\_kotschy): A

node 29 (anc. of terminals r\_baldschuanicus-r\_cassius\_n): A

node 30 (anc. of terminals r\_occidental-r\_uncinatus): B

node 31 (anc. of terminals r\_baldschuanicus-r\_uncinatus): AB

node 32 (anc. of terminals r\_stagnalis-r\_tembensis): C

node 33 (anc. of terminals r\_baldschuanicus-r\_tembensis): AC ABC

node 34 (anc. of terminals r\_acris-r\_tembensis): A

node 35 (anc. of terminals r\_acris-r\_constantino): A

node 36 (anc. of terminals r\_chius-r\_parviflo): A

node 37 (anc. of terminals r\_acris-r\_parviflo): A

node 38 (anc. of terminals r\_acris-r\_sericeus): A

node 39 (anc. of terminals r\_acris-r\_pinardi): A

### **2.3. arvensis clade**

**Areas**

**C: Circumboreal**

**W: West of Mediterranean**

**E: East of Mediterranean**

**I: Irano-Turanean**

**A: Central Asia**

**H: Himalaya and India**

>optimize;

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 9 0%

up & final on node: 6

optimization successful - exact solution

settings: maxareas=4, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 5 dispersals

optimal distributions at each node:

node 6 (anc. of terminals r\_brutius-r\_dissectus): I

node 7 (anc. of terminals r\_brutius-r\_caucasicus): I

node 8 (anc. of terminals r\_brutius-r\_sojakii): I

node 9 (anc. of terminals r\_arvensis-r\_sojakii): CWEIC

## **2.4. polyanthemios clade**

**Areas**

**E: Eurasia**

**N: North America**

**S: South America**

**F: Africa**

**W: Hawaii**

>optimize;

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 67 0%

up & final on node: 35

optimization successful - exact solution

settings: maxareas=5, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 15 dispersals

optimal distributions at each node:

node 35 (anc. of terminals *r\_fascicularis-r\_hispidus*): N  
 node 36 (anc. of terminals *r\_acriformis-r\_hispidus*): N  
 node 37 (anc. of terminals *r\_hawaiiensis-r\_mauiensis*): W  
 node 38 (anc. of terminals *r\_hawaiiensis-r\_petiolaris*): NW NSW  
 node 39 (anc. of terminals *r\_acriformis-r\_petiolaris*): N  
 node 40 (anc. of terminals *r\_acriformis-r\_septentrionali*): N  
 node 41 (anc. of terminals *r\_rarae-r\_oreophytus*): F  
 node 42 (anc. of terminals *r\_maclovianus-r\_orthorhynchus*): NS  
 node 43 (anc. of terminals *r\_diffusus-r\_orthorhynchus*): EN ES ENS  
 node 44 (anc. of terminals *r\_rarae-r\_orthorhynchus*): EF NF ENF EFS NFS ENFS  
 node 45 (anc. of terminals *r\_acriformis-r\_orthorhynchus*): N EN ENF ENS ENFS  
 node 46 (anc. of terminals *r\_acriformis-r\_tenuirostris*): E EN  
 node 47 (anc. of terminals *r\_macoounii-r\_repens*): EN  
 node 48 (anc. of terminals *r\_acriformis-r\_repens*): E  
 node 49 (anc. of terminals *r\_marginatus-r\_cornutus*): E  
 node 50 (anc. of terminals *r\_trilobus-r\_cornutus*): E  
 node 51 (anc. of terminals *r\_acriformis-r\_cornutus*): E  
 node 52 (anc. of terminals *r\_multifidus-r\_pinnatus*): F  
 node 53 (anc. of terminals *r\_acriformis-r\_pinnatus*): EF  
 node 54 (anc. of terminals *r\_polyanthemos-r\_serpens ssp. nemorosus*): E  
 node 55 (anc. of terminals *r\_sardous-r\_serpens ssp. nemorosus*): EN  
 node 56 (anc. of terminals *r\_sardous-r\_submarginatus*): E  
 node 57 (anc. of terminals *r\_sardous-r\_bulbosus*): E  
 node 58 (anc. of terminals *r\_sardous-r\_neapolitanus*): E  
 node 59 (anc. of terminals *r\_acriformis-r\_neapolitanus*): E  
 node 60 (anc. of terminals *r\_chinensis-r\_cantoniensis*): E  
 node 61 (anc. of terminals *r\_pensylvanicus-r\_silerifolius*): EN  
 node 62 (anc. of terminals *r\_chinensis-r\_silerifolius*): E  
 node 63 (anc. of terminals *r\_chinensis-r\_muricatus*): E ENF  
 node 64 (anc. of terminals *r\_peduncularis-r\_caprarum*): S  
 node 65 (anc. of terminals *r\_chinensis-r\_caprarum*): ED NFS ENFS  
 node 66 (anc. of terminals *r\_acriformis-r\_caprarum*): E ENC ENFS  
 node 67 (anc. of terminals *r\_acriformis-r\_bonariensis*): ES ENFS

## 2.5. Tethyan clade, upper part

### Areas

**C: Circumboreal**

**W: West of Mediterranean**

**E: East of Mediterranean**

**I: Irano-Turanean**

**A: Central Asia**

**H: Himalaya and India**

>optimize;

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 49 0%

up & final on node: 26

optimization successful - exact solution

settings: maxareas=4, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 10 dispersals

optimal distributions at each node:

node 26 (anc. of terminals r\_aduncus-r\_montanus): CW

node 27 (anc. of terminals r\_apenninus-r\_sartorianus): W

node 28 (anc. of terminals r\_apenninus-r\_pollinensis): W

node 29 (anc. of terminals r\_apenninus-r\_marschlinsii): W

node 30 (anc. of terminals r\_aduncus-r\_marschlinsii): W

node 31 (anc. of terminals r\_carinthiacus-r\_venetus): C

node 32 (anc. of terminals r\_pseudomontanus-r\_venetus): C

node 33 (anc. of terminals r\_gouanii-r\_venetus): CW

node 34 (anc. of terminals r\_villarsii-r\_venetus): W

node 35 (anc. of terminals r\_carpathicus-r\_venetus): CW

node 36 (anc. of terminals r\_aduncus-r\_venetus): W

node 37 (anc. of terminals r\_argyreus-r\_damascenus): I

node 38 (anc. of terminals r\_rumelicus-r\_sprunerianus): E

node 39 (anc. of terminals r\_psilostachys-r\_sprunerianus): E

node 40 (anc. of terminals r\_argyreus-r\_sprunerianus): IE

node 41 (anc. of terminals r\_macrorrhynchus-r\_millefolius): E

node 42 (anc. of terminals r\_hierosoly-r\_millefolius): E

node 43 (anc. of terminals r\_cicutarius-r\_millefolius): E

node 44 (anc. of terminals r\_argyreus-r\_millefolius): E

node 45 (anc. of terminals r\_illyricus-r\_heterorhizus): E  
node 46 (anc. of terminals r\_argyreus-r\_heterorhizus): E  
node 47 (anc. of terminals r\_garganicus-r\_millefoliatus): CWI  
node 48 (anc. of terminals r\_argyreus-r\_millefoliatus): CIW CWIE  
node 49 (anc. of terminals r\_aduncus-r\_millefoliatus): CWIE

## 2.6. Tethyan clade, lower part and combined with upper part

### Areas

**C: Circumboreal**

**W: West of Mediterranean**

**E: East of Mediterranean**

**I: Irano-Turanean**

**A: Central Asia**

**H: Himalaya and India**

optimizing...press command-period (Mac) or 'B' (Win) to stop

down on node: 49 0%

up & final on node: 26

optimization successful - exact solution

settings: maxareas=6, bound=250, hold=1000, weight=1.000, age=1.000

optimal reconstruction requires 22 dispersals

optimal distributions at each node:

node 27 (anc. of terminals r\_elbursensis-r\_termei): I

node 28 (anc. of terminals r\_aucheri-r\_termei): I

node 29 (anc. of terminals r\_afghanicus-r\_termei): I

node 30 (anc. of terminals r\_leptorrhynchus-r\_linearilobus): I

node 31 (anc. of terminals r\_afghanicus-r\_linearilobus): I

node 32 (anc. of terminals r\_afghanicus-r\_makaluensis): IH

node 33 (anc. of terminals r\_macropoioides-r\_papyrocarpus): I

node 34 (anc. of terminals r\_afghanicus-r\_papyrocarpus): I IH

node 35 (anc. of terminals r\_afghanicus-r\_regelianus): IA AF IAH

node 36 (anc. of terminals r\_upperPART\_regelianus): I WI CA WA CIA WIA CAH CIAH

node 37 (anc. of terminals r\_asiatricus-r\_gracilis): WI IE WIE

node 38 (anc. of terminals r\_upperPART-r\_gracilis): W I WI CIA CWIA CIAH CWIAH WIE CAE CWAE  
CIAE CWIAE CAHE CWAHE CIAHE CWIAHE

node 39 (anc. of terminals r\_gregarius-r\_bullatus): W  
 node 40 (anc. of terminals r\_olissiponensis-r\_paludosus): W  
 node 41 (anc. of terminals r\_olissiponensis-r\_pseudomillefol): W  
 node 42 (anc. of terminals r\_olissiponensis-r\_spicatus): W  
 node 43 (anc. of terminals r\_gregarius-r\_spicatus): W  
 node 44 (anc. of terminals r\_gregarius-r\_cortusifolius): W  
 node 45 (anc. of terminals r\_upperPART-r\_cortusifolius): W WI CWIA CWIAH CWAE CWIAE CWAHE  
 CWIAHE  
 node 46 (anc. of terminals r\_ambelyolobus-r\_cappadocicus): I IE  
 node 47 (anc. of terminals r\_ambelyolobus-r\_brachylobus): E IE  
 node 48 (anc. of terminals r\_ambelyolobus-r\_breyninus): I E CE CIE  
 node 49 (anc. of terminals r\_upperPART-r\_breyninus): WIE CWIE CWIAE CWIAHE

## Chapter 5

### Rapid speciation in high alpine and arctic species of *Ranunculus* during the Quaternary\*

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\* formatted for *Evolution*

## Abstract

The climatic oscillations and glaciations during the Quaternary had a strong impact on plant evolution. Here we study speciation processes in an arctic-alpine clade of *Ranunculus*. A phylogenetic study based on DNA sequences (ITS of nrDNA, *matK/trnK*, *psbJ-petA*) revealed a low genetic divergence among species, and a geographical grouping into four subclades (North America, southern Central Asia, northern Central Asian-Arctic and European lowland). Analysis of ecological and karyological data give insights into modes of speciation. In North America, the availability of a large area and a broad range of habitats triggered allopatric speciation and adaptive radiation. Habitat differentiation may have been enhanced by frequent polyploidy. In contrast, in the Himalayas and in Taiwan, the alpine species are restricted to a narrow ecological zone in high altitudes because of the lack of habitats in surrounding areas. A Neighbor Net analysis on an expanded sampling suggested recent gene flow; the predominance of one ploidy level (4x) may have weakened crossing barriers. The Arctic was colonized multiple times without a pronounced radiation. In the lowlands of temperate Europe, hybridization shaped the evolution of apomictic polyploid complexes. Ecological and geographical factors have strongly influenced the modes of Quaternary speciation in buttercups.

**Key words:** Adaptive radiation, allopatric speciation, apomixis, hybridization, North American Mountains, Himalayas, Arctic.

## Introduction

One of the most dramatic times of climatic change in Earth history is the Quaternary Period, which comprises ca. last 1.8 million years (Myr). This period was characterized by repeated global cooling and increasing advances of ice sheets and involving approximately 20 glacial advances and retreats (Wilson *et al.* 2000; Peizhen *et al.* 2001; Zachos *et al.* 2001). In the Northern Hemisphere, ice developed into temperate latitudes of North America and Eurasia ca. 2.75 million years ago (Ma; Willis and Niklas 2004). During the glacial periods, large areas of the northern Hemisphere were covered by ice. In North America, continental ice sheets extended at the Last Glacial Maximum (LGM) over much of the northern portion of continent. Most parts of Canada were ice-covered, including the shelf areas. The same can be said of Greenland. These areas preserve evidence of glaciations in the Neogene (Ehlers and Gibbard 2007). Climatic conditions were considerably drier and colder, and lowered sea levels exposed the Beringian land bridge, connecting the North American and Siberian land masses (Hopkins 1982). During full glacial advances, many boreal species persisted at lower latitudes south of the ice sheets, although some species may have been found along the north Pacific coast or in eastern Beringia (Central Alaska and far western Yukon, Lessa *et al.* 2003). In the Arctic most of northeast Russia and northwest Canada remained ice-free during Quaternary glaciations (Hulten 1937; Frenzel *et al.* 1992). Large areas of the northern parts of Russia and Siberia have repeatedly been affected by major glaciations during the Quaternary. Ice sheets that formed over Scandinavia spread eastwards across the northwest of the Russian Plains and the White Sea area (Svendsen *et al.* 2004). However, other authors assumed more limited ice caps over the Arctic Islands, the Polar Urals, the Central Siberian Uplands (Svendsen *et al.* 2004), and the Siberian Mountains (Ehlers and Gibbard 2007). The high mountains of Central Asia including the Himalaya, Hindu Kush and Karakoram Mountains constitute the glaciated areas outside of the polar regions (Owen *et al.* 2002b). The greatest glacial concentration occurred in the subtropical high mountains of the Greater Himalaya and in southern Tibet (Owen *et al.* 2002a).

The distribution and genetic diversity of plant species have been deeply modified by Pleistocene glaciations (Comes and Kadereit 1998; Abbott and Brochmann 2003; Hewitt 2004). During the climatic oscillations the species had to move, adapt or go extinct. Although, global cooling may have driven many taxa to extinction, it also may have been a major factor stimulating the diversification of others. The repeated isolation of plants and animals during intervals of

unfavorable climatic conditions had important evolutionary implications. In glaciated periods, advancing ice-shields either separated species as vicariant barriers or forced them to disperse to unglaciated areas. In the northern hemisphere, the extent of the ice sheets pushed the distribution of species to lower latitudes; however, large areas of northeast Asia and northwest America were not glaciated even at high latitudes (Abbott *et al.* 2000). During this southward migration, geographical barriers like big mountain chains and oceanic basins were important factors for speciation. The isolated populations diverged by vicariance, but contacted again during recolonization in postglacial phases (Barnosky 2005). Therefore climate fluctuation caused allopatric speciation through isolation, and hybridization after secondary contact. Although the climatic fluctuations may had the strongest effects in the glaciated areas, they also had a strong impact on the biota in adjacent regions by changing the biotic and abiotic environmental conditions in these areas.

Geographical isolation was long regarded as the most common mode of speciation by restricting gene flow between taxa (e.g., Nosil 2008). In sympatry, species can either evolve via ecological differentiation or other premating crossing barriers. There is growing evidence that ecological selection on traits such as morphology, environmental tolerance or reproductive timing has an important role for divergence and speciation (Schluter 2001). In plants, sympatric speciation is most commonly associated with hybridization and/or polyploidy. Homoploid hybrids can occupy new habitats that are extremely different from parental species via transgressive segregation (e.g., Rieseberg and Willis 2007). Polyploidy establishes an immediate crossing barrier against the parents, and causes dramatic genomic rearrangements accompanied by genetic and epigenetic changes (e.g., Soltis *et al.* 2004; Comai 2005; Chen 2007). “Genomic novelty” of polyploids may explain increased fitness; habitat differentiation and shifts in reproductive systems, thereby increasing the evolutionary potential (reviewed by Soltis *et al.* 2004; Brochmann *et al.* 2004; Mallet 2007).

The phylogeny and phylogeography of Paleoarctic and Neoarctic species in the Quaternary period is relatively well studied (e.g., Alsos *et al.* 2007). However, comparative studies on quaternary plant speciation in the whole area comparing different mountain system are scarce. Here we present a comprehensive data set from *Ranunculus* (Ranunculaceae) from North America, central and eastern Asia, and the Arctic. *Ranunculus* has approximately 600 species (Tamura 1993, 1995) and it has a worldwide distribution from the Tropics to the Arctic and the subantarctic zones. Species of *Ranunculus* are established in a variety of wet to dry habitats from

the lowland to high alpine zones and have evolved several morphological adaptations to different habitats (Paun *et al.* 2005; Hörandl and Emadzade in prep.). *Ranunculus* shows different levels of polyploidy, which is sometimes connected to apomixis (Hörandl *et al.* 2005, 2009). *Ranunculus* s. str. is currently considered to be composed of several well supported major clades (Hörandl *et al.* 2005; Paun *et al.* 2005; Gehrke and Linder 2009; Hoffmann *et al.* 2010; Emadzade *et al.* submitted). These studies revealed most arctic and alpine species of North America and Asia are nested in one clade (clade I in Hörandl *et al.* 2005; clade V in Paun *et al.* 2005; “North American-Eurasian high alpine” clade in Gehrke and Linder 2009; clade E in Hoffmann *et al.* 2010; clade IV in Emadzade *et al.* submitted). This clade comprises mainly perennial, small to medium-sized herbs (up to 40 cm high) with yellow flowers and swollen achenes; taxonomically it is treated as *R.* sect. *Auricomus* (Hörandl in press). The species are characterized by differences in leaf shape, indumentum, size and shape of petals, and shape of achenes. Interestingly, this clade included almost all alpine Himalayan (except *R. makaluensis* Kadota), Taiwanese, Central Asian, and North American species, some European lowland taxa, but no species from the European alpine system (except the North-American and arctic *R. pygmaeus* Wahlenb. that has also a small distribution area in the European Alps). The European representatives of this clade belong to the temperate to boreal *R. auricomus* group, which is an apomictic polyploid complex mainly distributed in lowland habitats (Hörandl 1998; Hörandl and Paun 2007). Much of the current distribution range of *Ranunculus* in alpine areas includes previously glaciated regions in the Rocky Mountains, Altai, Tien Shan, the Himalayas, and the Arctic. Surprisingly, the clade comprised no high alpine species from the southern hemisphere (Lockhart *et al.* 2001; Lehnbech *et al.* 2007; Hoffmann *et al.* 2010). Distribution ranges are also highly variable in this group, from very narrowly endemic (e.g., *R. anadyriensis*) to almost circumboreal and circumpolar (e.g., *R. nivalis* and *R. sulphureus*). Molecular dating approaches suggested origin and diversification of this clade already in the Middle Pliocene-Pleistocene (Paun *et al.* 2005; Emadzade and Hörandl, submitted), which probably was affected by Quaternary climatic fluctuations. Speciation in this clade has been influenced by reticulate evolution, hybridization and high frequencies of polyploidy (Hörandl *et al.* 2005, 2009). The *Ranunculus auricomus* clade provides an appropriate model system for studying the speciation and evolution of arctic-alpine plants in the northern hemisphere during the Quaternary period. To better understand of speciation in alpine systems, the study of phylogenetic relationships of species in the Himalayas can serve as a model system. The Himalayas are of special interest for studying speciation processes in alpine

floras, mainly because of their high elevation and central location within the Eurasian mountain belts. The Himalayas thus connect quite different floristic regions of Eurasia (Takhtajan 1986). In the altitudinal range, the Himalayas cover a subtropical belt, a temperate zone, an alpine and a high subnival and nival zone (e.g., Dobremez *et al.* 2008). The data from other genera rather resemble findings from studies on the Tibetan flora, where morphological differentiation and diversity seemed to be combined to generally poor molecular resolution of genetic differentiation (e.g., Liu *et al.* 2002, 2006; Wang and Liu 2004; Wang *et al.* 2005; Blösch *et al.* submitted), suggesting autochthonous origin and a rapid radiation.

To establish a sound phylogenetic framework as basis for a better understanding of arctic-alpine plant evolution, we generated and analyzed DNA sequence data from the nuclear ITS region as well as the plastid *matK/trnK* gene and the *psbJ-petA* intergenic spacer. These DNA regions have turned out to be most informative for *Ranunculus* in previous studies (Hörandl *et al.* 2005; Paun *et al.* 2005; Gehrke and Linder 2009; Hoffmann *et al.* 2010; Emadzade *et al.* submitted). We further compare ecological and karyological data to get insights into the main isolating factors that could explain the observed rapid speciation processes.

In this study we would like to (i) develop a phylogenetic framework for elucidating the processes of spatial and temporal diversification of arctic and alpine *Ranunculus* in the Northern Hemisphere, (ii) compare the evolutionary history in two different alpine systems, the North American Mountain chains and the Himalayas, (iii) understand whether rapid speciation in these areas was caused by adaptive radiation and ecological crossing barriers, and (iv) analyze the influence of hybridization and polyploidy on the evolution of taxa.

## Methods

### SAMPLING STRATEGY

In this study, new samples from North America, Central Asia, and Himalayas were added to earlier sequences obtained by Hörandl *et al.* (2005), Paun *et al.* (2005), and Hoffmann *et al.* (2010). To investigate speciation and assumed reticulate evolution in more detail new samples from the Himalayas, Kashmir, Tibet, the mountains of Taiwan and the Altai were collected. Determination of Himalayan species followed Kadota (1991), Riedl and Nasir (1991), Wang and Gilbert (2001), and was aided by additional herbarium collections from G. Miede (Hamburg, Germany), Bernhard Dickoré (Munich, Germany), and the herbarium of Edinburgh (U.K.).

Species determination was partly extremely difficult because of great variation in leaf shape and the scarcity of other diagnostic features. Some species of closely related clades (*Ranunculus sceleratus*, *R. fluitans*, *R. gmelinii*, *R. apiifolius*, *R. diffusus*, and *R. hispidus*) and genera, *Halerpestes*, *Cyrtorhyncha*, and *Beckwithia* (Paun *et al.* 2005; Hoffmann *et al.* 2010; Emadzade *et al.* submitted) were selected as outgroups. Voucher information and GenBank accession numbers are provided in Appendix 1.

## DNA-AMPLIFICATION AND SEQUENCING

Total genomic DNA from silica-dried or herbarium material was extracted using a modified CTAB technique (Doyle and Doyle 1987). The whole internal transcribed spacer region (ITS, including ITS1, the 5.8 gene, ITS2) was amplified as a single piece with primers ITS 18sF and ITS 26sR (Gruenstaeudl *et al.* 2009) or in the case of degraded DNA from poor quality herbarium tissue, in two pieces with additional primers (ITS 5.8sF and ITS 5.8sR) as internal primers (Gruenstaeudl *et al.* 2009). Sequencing of the *matK/trnK* region was performed according to the protocol described by Paun *et al.* (2005). Amplification of the non coding *psbJ-petA* region was carried out as a single piece in all samples by using primers of Shaw *et al.* (2007). PCR was performed in 23 µl reactions containing 20 µl 1.1× Reddy Mix PCR Master Mix (including 2.5 mM MgCl<sub>2</sub>; ABgene, Epsom, UK), 1 µl of 0.4% bovine serum albumin (BSA, Promega, Madison, WI, U.S.A.), and in the case of the ITS region, dimethyl sulfoxide (DMSO) to reduce problems associated with DNA secondary structure, 1 µl each primer (10 mmol/L) and 1 µl template DNA. PCR products were purified using *E. coli* Exonuclease I and Calf Intestine Alkaline Phosphate (CIAP; MBI-Fermentas, St. Leon8 Rot, Germany) according to the manufacturer's instructions. Cycle sequencing was performed using Big Dye<sup>TM</sup> Terminator v3.1 Ready Reaction Mix (Applied Biosystems), using the following cycling conditions: 38 cycles of 10 sec at 96°C, 25 sec at 50°C, 4 min at 60°C. All DNA regions were sequenced in both directions. The samples were run on a 3130xl Genetic Analyzers capillary sequencer (Applied Biosystems).

## SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

The sequences were aligned using Clustal X (Thompson *et al.* 1997). Subsequent corrections verified by eye using BioEdit version 7.0.9.0 (Hall 1999). Indels were treated as binary characters following the “simple indel coding method” (Simmons and Ochoterena 2000) using the program

SeqState version 1.36 (Müller 2005). Nuclear and chloroplast sequences were analyzed separately and combined. For most parsimonious (MP) analyses, we conducted heuristic searches with PAUP\* version 4.0b8 (Swofford 2002). The analyses involved 1000 replicates with stepwise random taxon addition, tree bisection–reconnection (TBR) and branch swapping saving no more than 10 trees per replicate. All characters were equally weighted and treated as unordered (Fitch 1971). Strict consensus trees were computed from all equally most parsimonious trees. Internal branch support was estimated using non-parametric bootstrapping (Felsenstein 1985) with 1000 replicates and 10 addition sequences replicates.

Bayesian Inference (BI) analysis was conducted using Mr.Bayes 3.1.2 (Ronquist and Huelsenbeck 2003). The best-fit substitution models were determined using Mr Modeltest 2.2 (Nylander 2004). Different partitions of the data set, ITS, *matK*, *trnK*, and *psbJ-petA*, were tested separately. A GTR+I+ $\Gamma$  substitution model was used for all partitions for final analysis. The MCMC setting for Bayesian analysis consisted of four runs with four chains each for 5,000,000 generations sampling every 1000<sup>th</sup> generations, using default priors and estimating all parameters during the analysis. Data from the first 1000 generations were discarded as the „burn-in” period, after confirming that likelihood values had stabilized prior to the 1000<sup>th</sup> generation. A majority rule consensus phylogeny and posterior probability (PP) of nodes were calculated from the remaining sample.

Since a non-hierarchical data structure has been inferred frequently in *Ranunculus* (Lockhart *et al.* 2001; Hörandl *et al.* 2005; Emadzade *et al.* submitted), we applied phylogenetic network methods (Huson and Bryant 2006) in the well-sampled Southern and Central Asian clade (clade II). We were interested to determine whether polytomies and low bootstrap support (BS) in this clade are due to conflicting support or absence of phylogenetic signal. To investigate the data structure in clade II, a Neighbor Net (NNet) analysis was performed using SplitsTree4 version 4.10 (Huson and Bryant 2006) applying Hamming distances with gaps and ambiguous sites coded as missing data. Neighbor Net calculates the support for phylogenetic “splits” (relationships) from genetic distances and displays these splits in a graph (i.e. a “splits graph” or “split network”). NNet uses an algorithm that determines a circular ordering of taxa (i.e., based on the extent of differences between their sequences the taxa are ordered around a circle). The layout on the circle determines what splits occur in the data and can be displayed in a planar graph. The support for each of these splits is then measured using a least squares method that adjusts the lengths of the splits in the splits graph so as to minimize the difference with the

pairwise distances in the original data matrix (Bryant and Moulton 2004; Huson and Bryant 2006). Non tree-like splits graphs indicate contradictory support for relationships and a non-hierarchical data structure. Bootstrap support for internal splits (which define clusters) was calculated with 1000 replicates.

## ECOLOGICAL AND KARYOLOGICAL FACTORS

To analyze different speciation processes in previously glaciated areas, we grouped the species into three main geographical regions (North America, southern Central Asian Mountains and Arctic-northern Central Asian Mountains). Arctic species that occur in more than one continent were assigned (*R. pygmaeus* and *R. nivalis*) to North America because of their phylogenetic placement and the large distribution in this continent. We compiled data describing altitude, habitats, and ploidy levels of species. Altitude was coded in nine categories (0 -  $\geq 4500$  m), the habitat preferences were summarized in seven categories: (1) subalpine-alpine tundra above treeline (including grassland, pastures, snowbeds, scree, rocks, glacier moraines), (2) arctic tundra, (3) steppe, grassland, prairies (all dry azonal vegetation, including sagebrush and semideserts), (4) coniferous forests, (5) broad-leaved forests, (6) meadows within the forest zone (including natural meadows in wetlands, but also man-made meadows), (7) riverine habitats and flood plains. Distribution, altitude, and habitat data of species were collected from current floras (e.g., Ovczinnikov 1937; Benson 1948; Meusel *et al.* 1965; Riedl and Nasir 1990; Tutin and Cook 1993; Rau 1993; Whittmore 1997; Wencai and Gilbert 2001; Dobremez *et al.* 2007; Borodina-Grabovskaya *et al.* 2007; Malyshev and Peschkova 2003) and personal observations in the field. Chromosome numbers and ploidy level were newly determined in three Himalayan species *Ranunculus hirtellus*, *R. palmatifidus*, and *R. rubrocalyx*. The other data were taken from Kuo (1990), Kurosawa (1971), Baack (2004), Hörandl *et al.* (2005), Hoffmann *et al.* (2010). All collected data are shown in Appendix 2.

## STATISTICAL ANALYSIS

We applied the Simple Matching method for pairwise comparisons of species with respect to distribution, zone and habitat. Then we compared matches among the three main areas that have been affected by glaciations (North America, South-Eastern Asia, Arctic and North Asia). We did not include the European group in these analyses, because of incomplete sampling of the temperate to arctic *Ranunculus auricomus* complex which comprises ca. 600 ecologically and

geographically differentiated microspecies (Hörandl 1998). Statistical significance of differences among areas was tested via a non-parametric Kruskal-Wallis test using SPSS. We did not perform statistical tests on karyological data because of too many missing data.

## Results

### MOLECULAR DATA AND PHYLOGENETIC RELATIONSHIPS

We amplified 595-600 bp of the ITS (ITS1, 5.8 rRNA, ITS2) region, 1376-1395 bp *matK* (*trnK/matK*), and 470-510 bp *psbJ-petA*. The combined aligned matrix included 3260 bp. Heuristic analyses of the ITS data set identified 2086 most parsimonious trees with a length of 388 steps (138 parsimony informative characters, consistency index [CI] = 0.6469, retention index [RI] = 0.7709, rescaled consistency index [RC] = 0.4987), while chloroplast markers (*matK/trnK*, *psbJ-petA*) revealed 6480 most parsimonious trees with 398 parsimony informative characters, CI = 0.7172 and RI = 0.8182. The MP analysis of combined data resulted in 6420 most parsimonious trees with CI = 0.6460 and RI = 0.7535. The ITS analysis did not resolve well relationships within species, showing a basal polytomy (not shown). The strict consensus tree of the chloroplast DNA overall showed better resolution, although, the basal polytomy still persisted (not shown). The topology provided by maximum parsimony (MP) of the combined data displays a better resolution than the results of either data set alone. The strict consensus tree is topologically very similar to the majority rule consensus tree from the Bayesian analysis, differences being only a few weakly supported nodes.

The topology provided by MP and BI analyses of the combined data displayed four weakly supported clades which largely represent geographical groups (Fig. 1a): I, North American, II, southern Central Asian, III, mainly European lowland, and IV, arctic-northern Central Asian species. Although the phylogenetic results presented here do not allow us to establish a detailed reconstruction of migration routes for this clade, they suggest a close relationship between taxa of the same geographical area. Since the genetic differentiation is in general very low, even a few homoplasious sites in the sequence data may cause a placement of a species outside the respective geographical group.

Clade I comprised all North American species except *Ranunculus micranthus*, which is nested in the European lowland clade (clade III), and *R. sulphureus* which has a circumpolar distribution and is related to Asian species (clade II). The Central Asian species *Ranunculus polyrhizus* is also nested within North American clade (clade I, Fig. 1a). Clade II includes all southern Central

Asian alpine species with a distribution from eastern Himalayas, eastern Hindu Kush, and Pamir to Tien Shan. Two alpine species from Taiwan, *Ranunculus formosomontanus* and *R. junipericola*, are also nested in this clade. All European lowland species form a clade (clade III) including the North American species *R. micranthus*, and two Asian species, *R. anadyriensis* endemic to northeastern Russia, and *R. krylovii*, endemic to the Altai Mountains. Clade IV included the Central Asian species with a main distribution in the Altai Mountains and around Baikal Lake, and the boreal to arctic species *R. affinis* (Fig. 1a).

NeighborNet analysis of an extended sampling of the southern Central Asian clade (II) revealed a highly reticulate structure of data with some moderately supported clusters emerging out of a basal network (Fig. 2). However, all species that are represented by more than one sample do not form distinct clusters, but appear multiple times in the Network (Fig. 2). For instance, *Ranunculus brotherusii* appears in five clusters (Fig. 2). *Ranunculus hirtellus*, *R. pulchellus* are each represented with five samples that are nested in two clusters. *Ranunculus membranaceus* with two samples from two regions (the eastern Himalayas and from Tibet), does not form a cluster. Three morphologically quite similar taxa, *R. longicaulis*, *R. longicaulis* subsp. *nephelogenes* and *R. pseudohirculus* form one cluster with 87% BS. The best supported cluster (BS = 99%) is that of *Ranunculus formosomontanus* and *R. junipericola* from the high mountains of Taiwan. Otherwise there is no obvious geographical grouping of samples within the NNet splits graph (Fig. 2).

## ECOLOGICAL AND KARYOLOGICAL DATA

The North American species showed a broad variation in all categories; in contrast, species of southern Central Asian Mountains occur only in a limited range of habitats and altitudes (Figs. 3a, b) and occur mainly in alpine/subalpine habitats, and around rivers. The arctic-northern Central Asian species occupied mainly alpine/subalpine, tundra and steppe-grassland habitats from the lowland to high altitudes. The simple matching analysis between the three characters altitude, zones and habitat showed that the southern Central Asian species have a great similarity in altitudes to each other, and differ in this factor significantly from the two other geographical groups. The North American species have the highest dissimilarity to each other but do not differ significantly from the Central Asian species group. The arctic-northern Central Asian species and the North American species occupy various ranges from low altitudes in the north to high altitudes in the south (Fig. 3a, Table 1). With respect to the habitat, all the comparisons between

geographical groups are significantly different. The southern Central Asian species are very similar to each other by occupying almost exclusively alpine habitats. The similarity is intermediate in the arctic-northern Central Asian species by growing mainly in alpine-sub alpine meadows, arctic tundra, and steppe-grassland. A large dissimilarity appears among the North American species, which differentiate over a broad range of habitats below and above the treeline (Fig. 3b, Table 1).

Karyological investigations in three species from Kashmir revealed *Ranunculus rubrocalyx* as diploid ( $2n = 16$ ), *R. hirtellus* as diploid and tetraploid ( $2n = 16, 32$ ), and *R. palmatifidus* as tetraploid ( $2n = 32$ ). In the seven southern Central Asian species with karyological information, the percentage of species with polyploid cytotypes is 86%, whereby 57% of polyploids in this group are tetraploid.

In the North American species, 50% of the 12 taxa with known chromosome numbers have polyploid cytotypes, whereby  $4x$  to more than  $9x$  cytotypes have been observed. In the arctic-Central Asian group, information on chromosome numbers is only available for four of ten species; three of them have polyploid cytotypes.

## Discussion

### HISTORICAL FACTORS

The low sequence divergence, low resolution of the phylogenetic trees and short internodes within this group of *Ranunculus* in comparison to the rest of the genus' phylogeny (Paun *et al.* 2005; Lehnbach 2008; Gehrke and Linder 2009; Hoffmann *et al.* 2010; Emadzade *et al.* submitted) suggest rapid and recent diversification of the *Ranunculus auricomus* complex in arctic and alpine areas. Previous age estimates based on DNA sequence data (Paun *et al.* 2005; Emadzade and Hörandl submitted) support a hypothesis that the *Ranunculus auricomus* clade has diversified in the Pleistocene or late Pliocene. At that time, the uplift of the Northern Hemispheric mountain chains was already advanced (Agakhanjanz and Breckle 1995) and should have allowed the migration of alpine species across these areas. On the other hand, this estimation coincides with climate fluctuations in the Quaternary. During cold periods, massive ice-sheet advanced into lower latitudes of North America and Eurasia, although, glaciations also occurred at more southern high mountain systems like the Alps, the Himalayas, and the Rocky Mountains. At each advance and retreat of ice, coastal distribution areas of species were exposed and submerged, respectively (Barnosky 2005). Despite of ice-free areas within northern and

Table 1. (a) Kruskal-Wallis test statistics for pairwise comparisons of species in North America, Himalaya, and arctic-central Asia with respect to habitat and altitude, and (b) results of significance tests.

A)

Area		N	Mean Rank	Mean Rank
			habitate	altitude
Similarity	North America	91	57.58	69.65
	Arctic-Central Asia	45	90.58	66.18
	Total	136		
Himalayas		66	116.6	105.16
North America		91	51.73	60.03
Total		157		
Himalayas		66	68.52	69.36
Arctic-Central Asia		45	37.64	36.41
Total		111		

B)

areas	Chi-Square		df		Asymp. Sig.	
	altitude	habitat	altitude	habitat	altitude	
	habitat					
North America & Arctic-Central Asia	21.991	0.240	1	1	<0.001	0.624
North America & Himalayas	80.992	38.671	1	1	<0.001	<0.001
Himalayas & Arctic-Central Asia	27.238	28.861	1	1	<0.001	<0.001

northeastern Asia, it can be assumed that Quaternary climate oscillations have affected the geographic distribution and evolution of species not only in the glaciated areas but also in the peripheral regions.

In North America the last ice sheet reached as far south as ca. 40° N (Hewitt 1996) and caused a southward displacement of climatic and vegetation zones, although the west coast of North America (Swenson and Howard 2005), some part of the northeastern of Canada, Southern Central Rocky Mountains were not so affected by glaciations (Hewitt 1996; Brunsfeld *et al.* 2001; Abbott and Brochmann 2003). In periods of glaciations, the north-south orientation of main Mountain chains did not block the migration of plants into southern regions and aided survival in lower

latitudes. The present distribution of arctic and alpine *Ranunculus* in North America could be explained by survival within the glacial refugia and northward migration from southern North America after ice-sheet retreat. When they radiated into ice-freed regions, they had a large area for dispersal and re-colonization. The north-south orientation of the Rocky Mountains and the Appalachians and a variety of different habitats (alpine tundra, subalpine parkland, forest steppe, temperate steppe and grassland; Ray and Adams 2001) enhanced ecological differentiation of lineages. Due to current distribution patterns of species in North America (Fig. 1b) allo-, parapatric and ecological speciation predominated and were probably caused by topographic fragmentation across the North American Mountain chains.

In contrast, in southern Asia, the southern range of the ice-sheet extended not so far south as in North America; however, the Tien Shan, the Himalayas, the Altai, and other Central Asian Mountains were glaciated in the Quaternary (Brigham-Grette 2001; Shi 2002). Because of the predominant east-west orientation of mountain chains, a hypothesis of a survival in southern refugia is less likely, and postglacial colonization patterns might have been more complex. Our data indicated that the Altai has been colonized two times independently: first, in clade IV, by species that are related to arctic species (*R. rigescens*, *R. lasiocarpus*), and second, in clade II by species that expand their distribution to the southern Asian mountains (*R. rubrocalyx*, *R. rufosepalus*, *R. longicaulis*). The presence of two different distribution patterns in the Central Asian Mountains could be due to the advance of Pleistocene cooling and the aridization accompanied by the Plio-Pleistocene mountain uplift in the Tien Shan and adjacent mountain ranges (Sun *et al.* 2004), or by a physical gap between the Altai and other mountains (Dsungarei). Alternatively, the Central Asian species in clade IV might be too young to have reached the southern mountain chains. The Central Asian Mountain species at the base of clade II could have migrated to the Himalayas along the mountain ranges that connect these two regions (Tien Shan and Pamir), and might be progenitors of the Himalayan species. In contrast to other Eurasian alpine genera, the *Ranunculus* species of the Himalayas do not show a closer relationship to those of the European Alps (see Kadereit *et al.* 2008). Rapid expansion into available habitats of Himalayas and adjacent mountain ranges could have been affected by geological events and climate fluctuation in Central Asia. The last phase of uplifting of Qinghai-Tibet plateau occurred ca. 1.6 Mya. This process led to a colder, drier climate and the formation of the modern river systems, and the establishment of a vegetation of alpine shrub, meadow, and expanded coniferous forests, which is favorable for buttercups (Fang *et al.* 1995; Shi *et al.* 1998).

## RAPID SPECIATION AND RETICULATE EVOLUTION IN THE HIMALAYAS

The Neighbour Net analysis does not show a pronounced clustering of the Himalayan samples into morphologically defined species. The splits graph does also not suggest a strongly hierarchical structure of the data, but rather indicates a basal network (Fig. 2). Such networks can result from hybridization, ancient lineage sorting, or horizontal gene transfer (e.g., Joly *et al.* 2009). The narrow reticulations indicate a low genetic distance and suggest an incomplete divergence within a relatively short time period, most likely within the Pliocene (Paun *et al.* 2005; Emadzade and Hörandl *et al.* submitted). Morphological studies (Hörandl and Emadzade in prep.) showed that characters are not stable and highly variable between the Himalayan species. The low sequence divergence between species (Fig. 2) and the reticulate data structure in the splits graph is probably due to ongoing gene flow (Nosil 2008). Gene flow is a strong homogenizing factor (Soltis and Soltis 2009) and might have obstructed the evolution of morphologically and genetically distinct taxa.

The Himalayan mountain chains were surrounded by areas unfavorable for buttercups from the Pleistocene up to now: deserts steppes in the north, and evergreen subtropical forests in the south, at least in the eastern parts of the Himalayas (Ray and Adams 2001). While deserts are in general too dry for alpine buttercups, subtropical forest floors are probably too shady.

Only a few species of *Ranunculus* occur in the southern slopes and the adjacent hillside regions of the Himalayas (*R. laetus*, *R. diffusus*, *R. ficariifolius*). However, these species belong to distantly related lowland clades of *Ranunculus* (Hoffmann *et al.* 2010; Emadzade *et al.* submitted). The same pattern applies to the high mountains of Taiwan, where the lowland vegetation is already tropical. In such narrow areas, the species did not have much possibility to expand their distribution (Fig. 3a, b). The concentration in high altitudes (Fig. 1a, b) and the lack of habitats suitable for buttercups in the lower altitudes enforced sympatry of populations above the treeline.

Our collected data showed predominant polyploidy, especially tetraploidy, in the Himalayan group (Fig. 1a; Appendix 2). A high potential for hybridization and polyploidization within sections of *Ranunculus* has been recorded before (Cook 1963; Lockhart *et al.* 2001; Hörandl *et al.* 2005). It has long been known that polyploidy is more prevalent at higher latitudes and altitudes (Hagerup 1932; Stebbins 1950; Felber 1991; Brochmann *et al.* 2004; Mable 2004), and

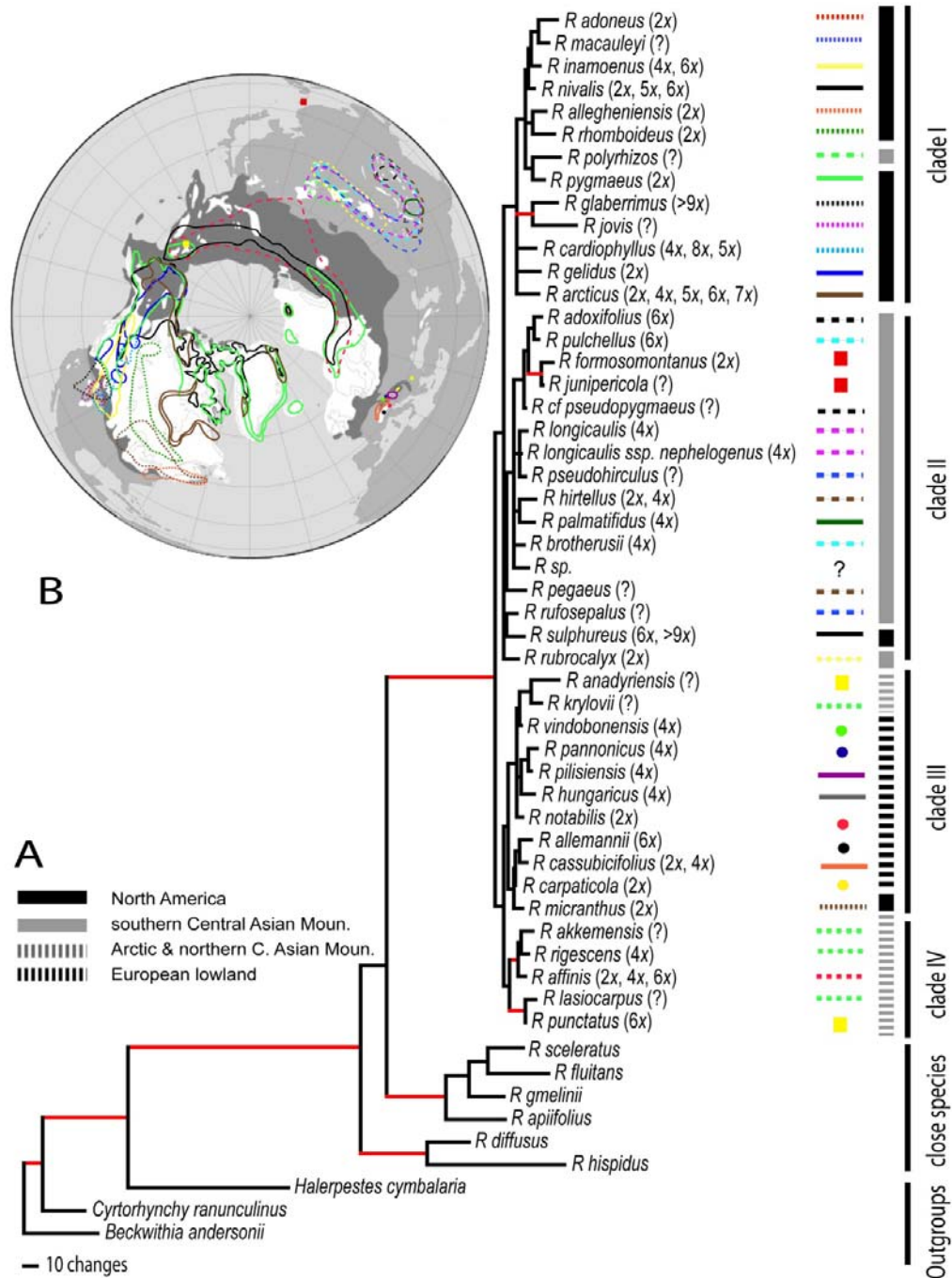


Figure 1. (A) Phylogenetic relationships of arctic-alpine-lowland *Ranunculus* species inferred from maximum parsimony and Bayesian analyses of ITS, *matK/trnK* and *psbJ-petA* data set. Branches with bootstrap support ( $\geq 90\%$ ) and posterior probability values ( $\geq 9.0$ ) are highlighted in red. Ploidy levels of species are indicated in parenthesis. (B) Distribution of arctic-alpine-lowland *Ranunculus* species used in this study. Distribution of ice shields (white) and tundra (dark grey) in the Northern Hemisphere at the last glacial maximum (modified from Abbott and Brochmann 2003). Each line indicates the current distribution of species. Species with a similar distribution share the type of line or symbol.

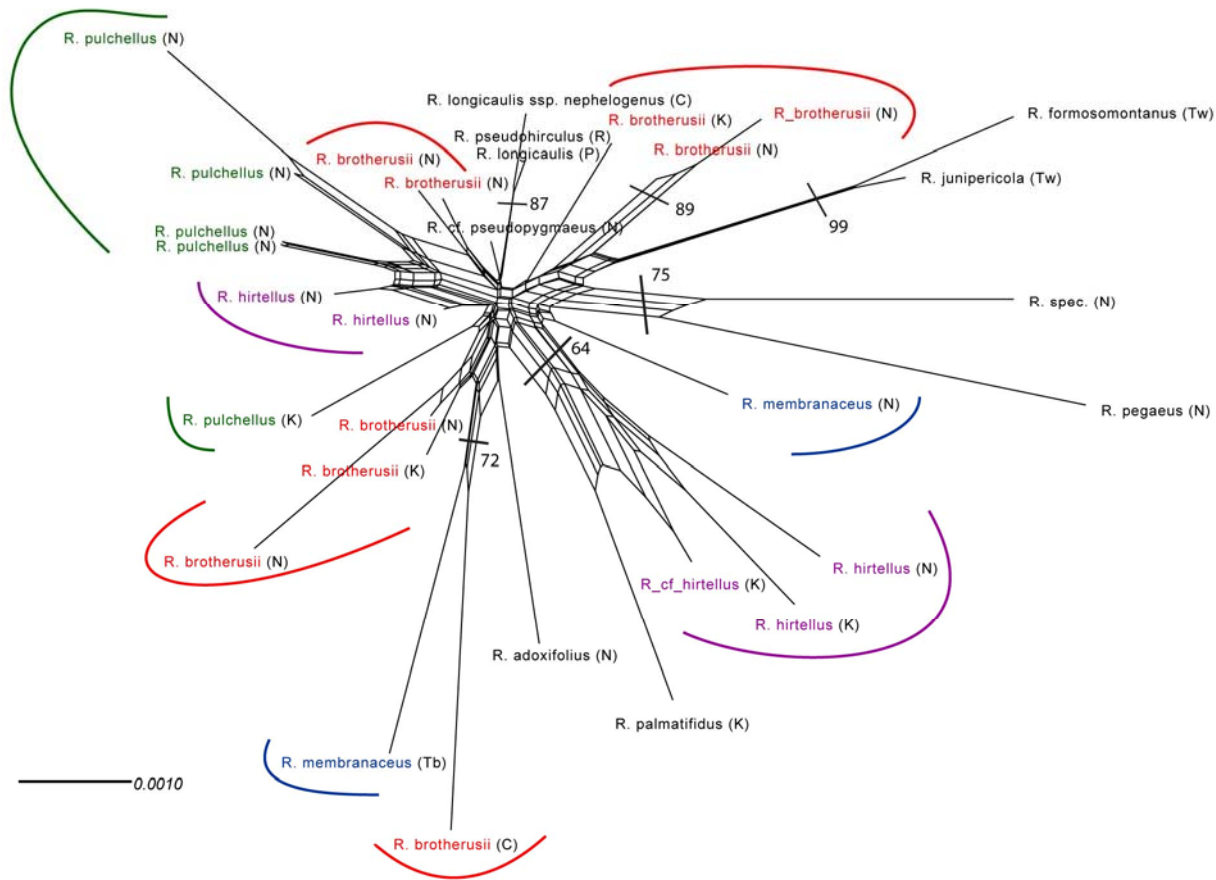


Figure 2. Neighbor Net split graph of Southern Asian species *Ranunculus* based on combined ITS, *matK/trnK* and *psbJ-petA* datasets. Clusters of samples belonging to the same morphotype are indicated by different colors. Locality of samples are indicated in parenthesis: C, China; K, Kashmir; N, Nepal; P, Pamir; R, Russia; Tb, Tibet; Tw, Taiwan. Bootstrap support values  $\geq 60$  are shown.

that allopolyploidy is common mechanism of diversification and sympatric speciation (e.g., Otto and Whitton 2000). Therefore in the first stage, restricted areas surrounded by unfavorable habitats (Ray and Adams 2001), may have caused sympatric distribution of buttercups after inter and postglacial migration to the Himalayas. Then hybridization caused the formation of allopolyploids, but high frequencies of tetraploids may have weakened the crossing barriers among polyploid taxa. Homoploidy in a sympatric area may enhance ongoing hybridization in the Himalayan species.

Therefore, the southern Asian species reflect young, perhaps still incomplete speciation events, which have been also observed in some taxa of the Tibetan flora (e.g., Liu *et al.* 2002, 2006;

Wang and Liu 2004; Wang *et al.* 2005). Liem (1990) believed that low sequence divergence and rapid radiation have been driven by low levels of competition in newly occupied habitats. But, in the case of Himalayan species, low sequence diversity and lack of distinct morphotypes could be an effect of hybridization and gene flow between species.

Relative high frequencies of aborted fruits in our Himalayan samples might be caused by hybridization, but also by apomixis, as it has been observed in the European *R. auricomus* complex (e.g., Hörandl 2008; Hörandl and Temsch 2009). Reticulate evolution in the Himalayan buttercups could be also connected to agamospermy, which has been documented in the “*R. auricomus* complex” (Hörandl 1998; 2002, 2008; Hörandl and Paun 2007; Hörandl *et al.* 2009). However, detailed information on breeding systems in the Himalayan species is still missing.

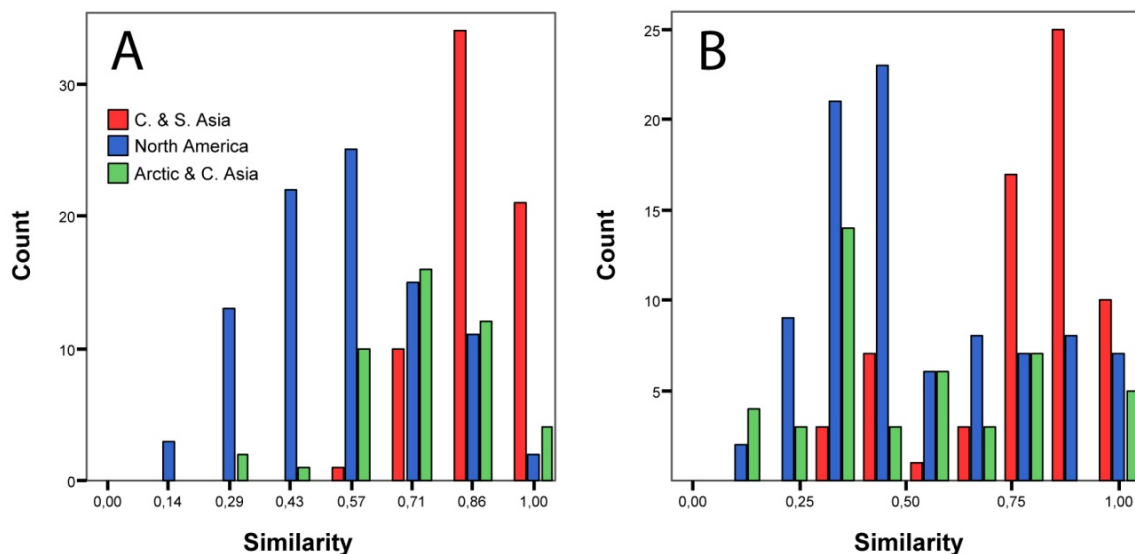


Figure 3. Histogram of results of Simple Matching method to compare pairwise similarity of species to each other with respect to habitat (A) and altitude (B) in the three different areas. Similarity = 0 indicates species pairs that are completely different, similarity = 1 indicates species pairs that totally match each other.

## ADAPTIVE RADIATION IN THE NORTH AMERICAN MOUNTAIN CHAINS

Rapid evolutionary radiation has been proposed to explain poorly resolved phylogenies in many groups of organisms (e.g., Whitfield and Lockhart 2007). Low genetic variation in arctic-alpine

species within a relatively short timeframe (ca. 2.5 Myr) might be correlated with the extensive habitat changes in the area that followed the final uplifting of the mountain and subsequent climatic oscillations in the Quaternary. These ecological shifts may not only have promoted rapid speciation, but may also have provided opportunities for speciation through polyploidization or hybridization (e.g., Marhold and Lihova 2006). Our data showed that half of the North American species have polyploid cytotypes. Polyploidy increases the variation for morphological, physiological and demographic traits relative to their diploid progenitors, and therefore enhances differentiation and partitioning of habitats among cytotypes (Soltis *et al.* 2004). In contrast to the polyploid complexes in the Himalayas, buttercups had in North America the opportunity to occupy not only various altitudes (Fig. 3a) across the mountain chains, but also different habitats in a large area (Ray and Adams 2001, Fig. 3b). Beside the alpine tundra, also subalpine parkland, forest steppes and the less shaded understory in temperate to boreal forests in the Rocky Mountains and the Appalachian mountains provided suitable habitats for buttercups. The dissimilarity of habitat preferences among species (Fig. 3b) confirms our hypothesis that ecological speciation was the main factor for speciation (Schluter 2000). Schluter (1996, 2000) believed that adaptive radiations are characterized by considerable taxonomic, morphological, and ecological diversity within a rapidly diversifying lineage. Probably the broad range of latitude, altitude, and habitats in North America provided opportunities for species to adapt to different environments (Hewitt 2004). A pronounced habitat differentiation in North America was also observed in other polyploid complexes (e.g., *Tolmiea menziesii*, Soltis and Soltis, 1989; *Antennaria rosea* complex, Bayer *et al.* 1991); a rapid speciation of recently formed polyploids has been documented in *Tragopogon* (Soltis *et al.* 2004). With respect to morphological diversification, we can confirm earlier authors (Benson 1948; Whittemore 1997) that the North American species represent distinct, easily diagnosable morphotypes. Phenotypic patterns do not suggest a pronounced hybridization, despite the observed low genetic divergence (Fig. 1a). Because of the broader range of habitats and elevation (Fig. 3a, b) occupied by North American species we suppose adaptive radiation in this group. Similar observations have been inferred from the alpine buttercups of New Zealand (Lockhart *et al.* 2001). However, more studies on phenotypic traits have to be conducted to understand the basis of adaptation (Schluter 2000). Ecological differentiation and also allopatric speciation (Fig. 1) may have established strong crossing barriers and limited hybridization among the North American buttercups.

## THE EUROPEAN LOWLAND CLADE: SPECIATION VIA APOMIXIS

In Europe massive ice sheets covered Fennoscandia, the Alps, Pyrenees, higher parts of the Carpathians (Messerli 1967). The phylogeographic patterns suggest that lowland organisms survived in three major glacial refugia, from where they migrated northwards after the glaciations (Schönswetter *et al.* 2005). Many of present-day alpine species colonized the European mountain chains from lower altitudes and latitudes (Hewitt 2004). Indeed, a main question in our study is why did European *Ranunculus* species in our investigated clade rarely reach the alpine zone and has not diversified there? One reason could be the competition between this group and European alpine species from other clades: first, a group of European white flowering species (*R.* sects. *Leucoranunculus*, *Aconitifolii*, *Ranuncella* sensu Hörandl in press) occupy a broad range of habitats from the forest zone up to the glacier regions. These taxa appeared either as basal or in unresolved positions at the backbone of the *Ranunculus* phylogeny (Hörandl *et al.* 2005; Paun *et al.* 2005; Hoffmann *et al.* 2010, Emadzade *et al.* submitted). Divergence times for these clades range from 5.5 to 1.1 Mya in Paun *et al.* 2005 (their clades I-III), and 12.0 to 1.0 Mya in Emadzade and Hörandl submitted. Second, the distantly related yellow-flowered *R. montanus* group (*R.* sect. *Euromontanus*, Hörandl in press) which belongs to the Tethyan clade (Emadzade *et al.* submitted) is widely distributed in subalpine and alpine grassland habitats (0.4 My old in Paun *et al.* 2005, 3.5 My in Emadzade and Hörandl submitted). Therefore, the European Alps may have been colonized multiple times by other *Ranunculus* species, and suitable habitats may have already been occupied.

The apomictic *Ranunculus auricomus* polyploid complex is nested within this clade, as observed in all previous phylogenetic studies. Hörandl *et al.* (2005, 2009) have given evidence for reticulate relationships and hybridization within this group. The diploid sexual species may have evolved via allopatric speciation in periglacial areas, and may have formed apomictic taxa via secondary contact hybridization (Paun *et al.* 2006; Hörandl *et al.* 2009). With the exception of a few agamospecies that reach the subalpine-alpine zone (*R. allemannii*, *R. melzeri*), the complex is mostly distributed in forest and meadow habitats at lower altitudes. Because of the incomplete sampling of the complex in the present study, we refrain here from a detailed analysis; a more complete discussion of the *R. auricomus* complex has been presented elsewhere (Hörandl 1998; Hörandl and Greilhuber 2002; Hörandl and Paun 2007; Hörandl *et al.* 2009).

## MULTIPLE COLONIZATION AND LIMITED SPECIATION IN THE ARCTIC

The arctic-northern Central Asian group is the least homogeneous geographical group, because arctic species fall not only in clade IV, but appear also in all the other clades. This pattern fits to a scenario of multiple colonization of the Arctic as observed in the whole genus *Ranunculus* (Hoffmann *et al.* 2010). However, a pronounced radiation seems to be missing in the Arctic. With respect to altitude and habitat, the differentiation among species is lower than in North America, but higher than in the southern Asian mountains. The arctic tundra provides a large area with habitats that are in general appropriate for buttercups. Therefore, this region provided probably opportunities for rapid dispersal and postglacial colonization, but to a lesser extent for rapid speciation. A more detailed discussion of the arctic species has been provided by Hoffmann *et al.* (2010).

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**Appendix 1**, Species sampled, voucher information and GenBank accessions of DNA sequences analyses in this study (BG: Botanical garden).

Taxon	Country; collector, collection No.; Herbar	GenBank accession Nos.		
		ITS	matK/trnK	psbJ-petA
<i>Beckwithia andersonii</i> (A. Gray) Jeps.	cult. Gothenburg BG; Johansson s.n.; GB	AY680197	AY954238	GU258003
<i>Cyrtorhyncha ranunculina</i> Nutt. ex. Torr. & A. Gray.	USA; Nunn 1775; RM	GU257973	GU257981	GU258005
<i>Halerpestes cymbalaria</i> (Pursh) Greene	cult. Rezia BG; Johansson 204; LD	AY680196	AY954237	GU258006
<i>R. adoneus</i> A. Gray	USA, Colorado; Ehrendorfer FER70; WU	AY680030	+	USA; Tremetsberger s.n.; WU; +
<i>R. adoxifolius</i> Hand.-Mazz.	Nepal; Hörandl & Emadzade 9677a; WU	+	+	+
<i>R. affinis</i> R. Br.	Russia; Doronkin & Kulagina 076; NSK	FM242811	FM242747	
<i>R. akkemensis</i> Polozhij & N.V.Revyakina	Russia; Tribsch 9605; WU	+	+	+
<i>R. allegheniensis</i> Britton	USA; Keener 2004-2; WU	+	+	+
<i>R. allemannii</i> Br.-Bl.	Austria; Hörandl 6687; WU	AY680039	+	+
<i>R. anadyriensis</i> Ovcz.	Russia; Savenkov et al., 180; LE	FM242802	FM242738	
<i>R. apiifolius</i> Pers.	Chile; Lehnebach s.n.; VALD	AY680092	AY954140	Uruguay; Lorentz 533W; GU258016
<i>R. arcticus</i> Richardson	cult. Devonian BG; Johansson 239; LD	AY680049	AY954125	+
<i>R. brotherusii</i> Freyn.	China; X. Zhao 28417; MPN	AY680055	+	+
<i>R. brotherusii</i> Freyn.	Pakistan; Lone 1752; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Pakistan; Lone 1751; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Hörandl & Emadzade 9680; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Hörandl & Emadzade 9686; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Hörandl & Emadzade 9702; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Hörandl & Emadzade 9665; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Hörandl & Emadzade 9678; WU	+	+	+
<i>R. brotherusii</i> Freyn.	Nepal; Staudinger 484280; LI	AY680037	AY954119	+
<i>R. cardiophyllus</i> Hook.	cult. Gothenburg BG; Johansson HZ 86-29	AY680045	AY954124	+
<i>R. carpaticola</i> Soó	Slovakia; Hörandl 8483; WU	AY680041	AY954111	FJ619866
<i>R. cassubicifolius</i> W. Koch	Germany; Hörandl 8476; WU	AY680040	AY954112	FJ619867
<i>R. cf. hirtellus</i> Royle	Pakistan; Lone 1757; WU	+	+	+
<i>R. cf. pseudopygmaeus</i> Hand.-Mazz.	Nepal; Hörandl & Emadzade 9689; WU	+	+	+
<i>R. diffusus</i> DC.	Nepal; Hörandl & Emadzade 9706; WU	+	+	+
<i>R. fluitans</i> Lam.	Sweden; Johansson s.n. ; —	AY680069	AY954129	+
<i>R. formosomontanus</i> Ohwi	Taiwan; Hörandl 9548; WU	+	+	+
<i>R. gelidus</i> Kar. & Kir.	Xinjiang, China; Wang 28426; MPN	AY680054	AY954114	+
<i>R. glaberrimus</i> Hook.	USA; Lyall 1861; ZT	+	+	+
<i>R. gmelinii</i> ssp. <i>gmelinii</i> DC.	U.S.A., Alaska; Schröck 454907; LI	AY680063	AY954128	
<i>R. hirtellus</i> Royle	Pakistan; Lone 1756; WU	+	+	+
<i>R. hirtellus</i> Royle	Nepal; Hörandl & Emadzade 9685; WU	+	+	+
<i>R. hirtellus</i> Royle	Nepal; Hörandl & Emadzade 9660; WU	+	+	+
<i>R. hirtellus</i> Royle	Nepal; F. Tod 372997; LI	AY680038	AY954120	+
<i>R. hispidus</i> Michx.	USA, Pennsylvania; Keener 2004-3b; WU	+	+	+
<i>R. hungaricus</i>	Hungary; Dunkel 20735; WU	FJ619892	FJ625805	FJ619881
<i>R. inamoenus</i> Greene	USA, Utah; Albach 842; WU	+	+	+

<i>R. jovis</i> A. Nelson	USA; Tepe 2465; Utah	+	+	+
<i>R. junipericola</i> Ohwi	Taiwan; Hörandl 9547; WU	+	+	+
<i>R. krylovii</i> Ovcz.	Russia; V. Totov s.n.; LE	FM242826	FM242762	
<i>R. lasiocarpus</i> C.A. Mey.	Russia; V. Zuev 429; LE	FM242813	FM242749	
<i>R. longicaulis</i> var. <i>nephelogenus</i> Edgeworth	?; Q. Zheng 28420; MPN	AY680052	+	+
<i>R. longicaulis</i> C.A.Mey.	Pakistan; Millinger 470564; LI	AY680051	AY954117	+
<i>R. macauleyi</i> A. Gray	USA; - 705285; RM	+	+	+
<i>R. membranaceus</i> Royle	Nepal; Hörandl & Emadzade 9696; WU	+	+	+
<i>R. membranaceus</i> Royle	Tibet; Q. Zheng 28419; MPN	AY680056	+	+
<i>R. micranthus</i> Nutt.	USA; Lonsing 50563; LI	AY680042	AY954113	+
<i>R. nivalis</i> L.	Sweden; Johansson s.n.; -	AY680046	AY954123	GU258032
<i>R. notabilis</i> Hörandl & Guterm.	Austria; Hörandl 5612; WU	AY680033	AY954115	FJ619873
<i>R. palmatifidus</i> Riedl	Pakistan; Lone 1763; WU	+	+	+
<i>R. pannonicus</i> Soó	Austria; Hörandl 5564; WU	AY680032	+	+
<i>R. pegaeus</i> Hand.-Mazz.	Nepal; Hörandl & Emadzade 9695; WU	+	+	+
<i>R. pilisiensis</i> Soó	Hungary; Hörandl 6600; WU	AY680034	+	+
<i>R. polyrhizos</i> Steph. ex Willd.	Russia; Kuznetsov n.s.; LE	FM242839	FM242775	
<i>R. pseudohirculus</i> Schrenk ex Fisch. & C. A. Mey.	Russia; Tribsch 9593; WU	AY680111	AY954118	
<i>R. pulchellus</i> C. A. Mey.	Pakistan; Lone 1764; WU	+	+	+
<i>R. pulchellus</i> C. A. Mey.	Nepal; Hörandl & Emadzade 9670; WU	+	+	+
<i>R. pulchellus</i> C. A. Mey.	Nepal; Hörandl & Emadzade 9679; WU	+	+	+
<i>R. pulchellus</i> C. A. Mey.	Nepal; Hörandl & Emadzade 9671; WU	+	+	+
<i>R. pulchellus</i> C. A. Mey.	Nepal; Hörandl & Emadzade 9687; WU	+	+	+
<i>R. pulchellus</i> C. A. Mey.	Nepal; Hörandl & Emadzade 9679; WU	+	+	+
<i>R. punctatus</i> Jurtzev	Russia; Zimarskaya & al. s.n., LE	FM242818	FM242754	+
<i>R. pygmaeus</i> Wahlenb.	Sweden; Larson & Granberg 9345; WU	AY954242	AY954122	+
<i>R. rhomboideus</i> Goldie	USA; Hezns et al. s.n.; LE	FM242854	FM242790	
<i>R. rigescens</i> Turcz. ex Osten-Sack. & Rupr.	Russia; Malyshev & Barzunov n.s.; LE	FM242809	FM242745	
<i>R. rubrocalyx</i> Kom.	Russia; Kaletkina s.n.; M	+	+	+
<i>R. rufosepalus</i> Franch.	Pakistan; Millinger392897; LI	AY680047	AY954121	GU258042
<i>R. sceleratus</i> L.	Iran; Emadzade 112; WU	GU257971	GU257993	GU258043
<i>R. spec.</i>	Nepal; Hörandl & Emadzade 9694; WU	+	+	+
<i>R. sulphureus</i> Solande.	Russia; R. Elven & H. Solstad 20; WU	+	+	+
<i>R. vindobonensis</i> Hörandl & Guterm.	Austria; Hörandl 6602; WU	AY680035	+	+

+ Sequences which will be submitted to GenBank for publishing.

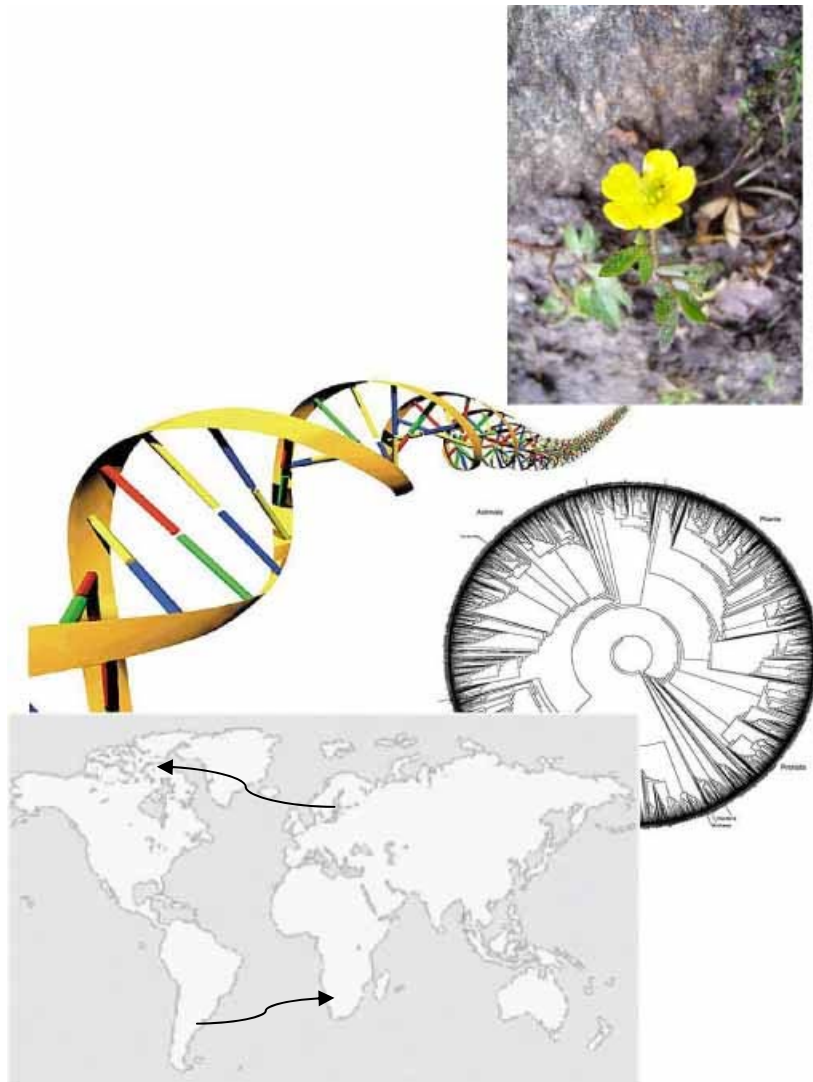
Appendix 2, Collected data including habitat, altitude, and ploidy level (\*Southern Central Asian species, ●North American species, ‡Arctic and Northern Central Asian species).

	Habitat							Altitude									Ploidy level							
Taxa	Subalp.-alpine meadows	Arctic tundra	Steppe, grassland, prairies	Coniferous forest	Broad-leaved forest	Meadows (within forest zone)	Riverine, flood plains	0-500	500-1000	1000-1500	1500-2000	2000-2500	2500-3000	3000-3500	3500-4000	4000-up	2x	4x	5x	6x	7x	8x	9x	
<i>R. adoxifolius</i> *	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	
<i>R. brotherusii</i> *	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	
<i>R. formosomontanus</i> *	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	
<i>R. pegaeus</i> *	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	-	-	-	-	-	-	-
<i>R. hirtellus</i> *	1	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	
<i>R. junipericola</i> *	1	0	0	1	0	0	0	0	0	0	1	1	1	1	1	1	-	-	-	-	-	-	-	
<i>R. longicaulis</i> *	1	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	
<i>R. membranaceus</i> *	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	-	-	-	-	-	-	-	
<i>R. palmatifidus</i> *	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	
<i>R. pseudohirculus</i> *	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	-	-	-	-	-	-	-	
<i>R. pseudopygmaeus</i> *	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	-	-	-	-	-	-	-	
<i>R. pulchellus</i> *	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	
<i>R. adoneus</i> ●	1	0	0	0	0	0	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	
<i>R. allegheniensis</i> ●	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
<i>R. cardiophyllus</i> ●	0	0	0	1	1	1	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	1	0	
<i>R. gelidus</i> ●	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	
<i>R. glaberrimus</i> ●	0	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	
<i>R. inamoenus</i> ●	1	0	0	1	1	1	0	0	0	0	1	1	1	1	0	0	0	1	0	1	0	0	0	
<i>R. jovis</i> ●	1	0	0	1	1	0	0	0	0	0	1	1	1	0	0	0	-	-	-	-	-	-	-	
<i>R. macauleyi</i> ●	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	-	-	-	-	-	-	-	
<i>R. micranthus</i> ●	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
<i>R. nivalis</i> ●	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0	
<i>R. arcticus</i> ●	1	1	0	0	0	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	
<i>R. pygmaeus</i> ●	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	
<i>R. rhomboideus</i> ●	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	
<i>R. sulphureus</i> ●	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	
<i>R. affinis</i> ‡	0	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1		1	0	0	0	
<i>R. akkemensis</i> ‡	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	-	-	-	-	-	-	-	
<i>R. anadyriensis</i> ‡	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	-	-	-	-	-	-	-	
<i>R. krylovii</i> ‡	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	-	-	-	-	-	-	-	
<i>R. lasiocarpus</i> ‡	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	-	-	-	-	-	-	-	
<i>R. polyrhizos</i> ‡	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	-	-	-	-	-	-	-	
<i>R. punctatus</i> ‡	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	
<i>R. rubrocalyx</i> ‡	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	
<i>R. rufosepalus</i> ‡	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	-	-	-	-	-	-	-	
<i>R. rigescence</i> ‡	1	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	

## Appendixes

### Abstracts of Contributions to International Conferences

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## **Phylogenetic relationships, biogeography and morphological adaptations of *Ranunculus* in southern Eurasia**

Khatere Emadzade & Elvira Hörandl

*The sixth Biennial Conference of the Systematics Association, 28-31 August 2007, Royal Botanic Garden Edinburgh*

*Ranunculus* L. (Ranunculaceae) comprises c. 600 species and it is distributed worldwide in all continents. *Ranunculus* has a center of diversity and high degree of endemism in the Mediterranean and Irano-Turanian region, with special adaptations to the summer-dry climate such as life form, underground parts and shape of fruits. Previous studies on related European taxa (Hörandl & al., 2005; Paun & al., 2005) show that phylogenetic relationships in these groups are unclear and contradict all previous classifications.

To understand phylogenetic relationships, evolution and adaptation of morphological characters to certain environmental conditions, to identify characters that are useful for classifications, and to understand geographical differentiation patterns in these regions are the main aim of our study. A molecular phylogeny based on DNA sequences of the nuclear ITS region and the plastid *matk/trnk* region are established. Sequences of both markers are analyzed using maximum parsimony in separate and combined analyses. The results were compared with morphologically-based taxonomic treatments.

The species of the meridional and temperate zones are concentrated in a well supported clade (100% bootstrap), including the former genus *Gampsoceras pinardii* (= *R. pinardi*) with various autapomorphic nucleotide substitutions. *R. pinardi* is an annual species with flat spiny fruits similar to *R. arvensis* and big fruits with very long beaks similar to *Ceratocephala*. It is nested within *Ranunculus* and not confirmed as a separate genus. It is neither a sister of *R. arvensis* nor of *Ceratocephala*, the position of this species is unclear. The topology of this clade in the combined analysis shows several well supported geographical subclades in the meridional zone. It shows high degrees of speciation in these regions which arises from the variety of climates in this area, especially in the Irano-Turanian region. The clades of temperate zones are

heterogeneous; this may be due to overlap of distributions areas of species during the evolution of the genus, or lack of extinct ancestral species in the dataset or incomplete collection in the region. The morphological characters such as life form, shape of leaves, and shape of achenes are highly homoplasious in this group.



**SYSTEMATICS 2008**  
7-11 April 2008, Göttingen



## **Molecular phylogeny, biogeographical history and a revised classification of *Ranunculus* s.l. (Ranunculaceae)**

Khatere Emadzade, Elvira Hörandl, Carlos Lehnebach & Peter Lockhart

*10<sup>th</sup> Annual Meeting of the Gesellschaft für Biologische Systematik*

*18<sup>th</sup> International Symposium "Biodiversity and Evolutionary Biology" of the German Botanical Society*

*Ranunculus* s.l. comprises c. 600 species and is distributed in all continents. Phylo-genetic reconstruction using DNA sequences (nrITS, matK-trnK) and morphological characters were utilized to get insights into relationships and evolutionary traits of the genus. Combined molecular data of c. 200 species reveal a large core clade comprising *Ranunculus* s.str., excluding the small genera *Laccopetalum*, *Krapfia*, *Ceratocephala*, *Myosurus*, *Ficaria*, *Coptidium*, *Beckwithia*, *Cyrtorhyncha*, *Halerpestes*, *Peltocalathos*, *Callianthemoides*, and *Arcteranthis*, but including the water-buttercups and the monotypic genus *Aphanostemma*. Biogeographical analyses of *Ranunculus* s.str. suggest a strong radiation within the Mediterranean - Irano-Turanian region, supporting the existence of an ancient Tethyan area. The Himalayan species are related to arctic-circumpolar, C. Asian and N. American, and European high mountain taxa. At least two independent eastern Asian - North American disjunctions including endemic species of Hawaii, are observed. Altogether the biogeographical history of the genus is in all continents not only shaped by multiple colonization events, but also by rapid regional diversifications. Morphological analyses suggest a high adaptive potential of structures, especially in vegetative parts; they show high levels of homoplasy and are not useful for classifications. Characters of fruits and petals, but also karyological features are more conserved and support not only the circumscription of genera within Ranunculeae, but also major clades within *Ranunculus* s.str. An outline of a completely new sectional classification is presented.

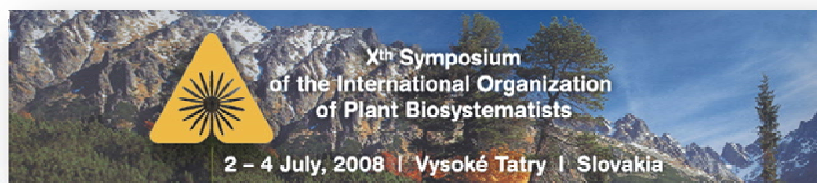


## **Phylogenetic Relationships, Evolution and Biogeography of *Ranunculus* (*Ranunculaceae*) in the Northern Hemisphere**

Khatere Emadzade & Elvira Hoerandl

*Annual Meeting of the Society for Molecular Biology and Evolution. 5 -8 June, 2008, Barcelona*

Ranunculus comprises c. 600 species worldwide with a center of diversity in meridional and submeridional zones. To understand phylogenetic relationships, evolution, and geographical differentiation patterns in the northern hemisphere, we constructed a molecular phylogeny based on DNA sequences of the nuclear ITS region and the plastid matk/trnk regions using Maximum parsimony and Bayesian inference in separate and combined analyses. Biogeographical analysis was implemented using the program DIVA. Comparing the trees base on ITS and plastid markers and split decomposition analysis shows incongruence in some clades and taxa that may be due to hybridization. The combined analysis shows several well supported geographical subclades in the meridional zone. It shows high degrees of speciation in this region which arises from the variety of climates in this area. The clades of temperate zones are heterogeneous; this may be due to overlap of distributions areas of species during the evolution of the genus, or lack of extinct ancestral species in the dataset. Biogeographical analyses of Eurasian taxa show different origin of Mediterranean alpine species, west and center Asian mountains and disjunctions between North American and East Asian species. Rapid adaptive speciation, reticulate evolution and extreme dispersal shape the evolution of the genus.



## Phylogenetic and biogeographical studies of alpine species of *Ranunculus* in Eurasia

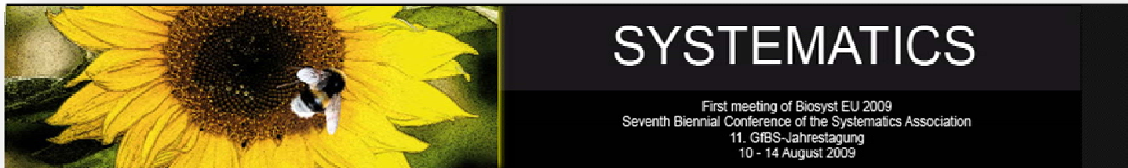
Khatere Emadzade & Elvira Hörandl

*Xth Symposium of the International Organization of Plant Biosystematists, 2-4 July 2008, Vysoké Tatry, Slovakia*

*Ranunculus* is the largest genus in Ranunculaceae. It comprises c. 600 species and is distributed worldwide in all continents. Morphological adaptations enable the genus to colonize a very broad spectrum of habitats, ranging from terrestrial to aquatic, arctic or alpine areas. The genus has a great diversity of species in the Eurasian mountains. Previous studies on European taxa (Hörandl & al., 2005; Paun & al., 2005) suggested that European alpine species have mostly an autochthonous origin, but the relationships to the species of the Asian mountains remained unclear.

A molecular phylogeny based on DNA sequences of the nuclear ITS region and the plastid *matK/trnK* region has been worked out to establish the ecological and geographical clades in a worldwide framework. Biogeo-graphical analysis was implemented using the program Dispersal – Vicariance Analysis (DIVA).

Biogeographical analyses of genus reveal a radiation within the ancient Tethyan area. Tethyan's taxa originated in the meridional zone and migrated to temperate and boreal zones. The mountain species of the Mediterranean and western Irano-Turanian region (East to Hindu Kush) have the same origin but are not related to the species of central Asian mountains (Altai, Himalaya, Tien-shan, Tibet). The central Asian high mountain species are related to arctic, northern European and North American species. This clade originated probably in North America and migrated via a northern route to Eurasia. This geographical differentiation within Eurasia might be due to different climates, but also to speciation and diversification of clades in different time periods.



## **Molecular phylogeny, Evolution and Biogeography of *Ranunculus* s. l. (Ranunculaceae)**

Khatere Emadzade & Elvira Hörandl

*1<sup>st</sup> meeting of Biosyst EU 2009 & 7<sup>th</sup> Biennial Conference of the systematic Association, 10-14 August 2009,  
Netherland, Leiden.*

*Ranunculus* comprises c. 600 worldwide distributed species. Phylogenetic reconstruction using DNA sequences (nrITS, matK-trnK, psbJ-petA) and morphological characters were utilized to get insights into relationships and evolutionary traits. Combined molecular data of c. 200 species reveal a large core clade comprising *Ranunculus* s.str., excluding several genera. Biogeographical analysis of genera suggests a northern hemispheric origin of the tribe and multiple colonization of the S. hemisphere, and reveals a strong radiation of *Ranunculus* within the Mediterranean-Irano-Turanian region. The mountain species of the Mediterranean and western Irano-Turanian region have the same origin but are not related to the species of central Asian mountains. The Himalayan species are related to arctic-circumpolar, C. Asian and N. American, and European high mountain taxa. At least two independent eastern Asian- North American disjunctions are observed. Altogether the biogeographical history of the genus is in all continents not only shaped by multiple colonization events, but also by rapid regional diversifications. Morphological analyses suggest a high adaptive potential of structures. Characters of fruits and petals, but also karyological features are more conserved and support not only the circumscription of genera within Ranunculeae, but also major clades within *Ranunculus* s.str. An outline of a new sectional classification is presented.

## Curriculum Vitae

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### ➤ **Educational Qualification**

- |           |   |
|-----------|---|
| 2006      | Ph.D. Studies in Botany, Systematic and Evolutionary of Botany, Faculty of Life Sciences, University of Vienna.<br><b>Ph. D. Thesis:</b> Phylogenetic Relationships, Evolution and Biogeography of <i>Ranunculus</i> (Ranunculaceae) and allied genera. |
| 1997-2000 | M. Sc. in botany (plant systematics and ecology), Ferdowsi University of Mashad, Iran.<br><b>M.Sc. Thesis:</b> Taxonomical study of <i>Anthemis</i> and <i>Matricaria</i> (Asteraceae) in east of Iran based on morphological and anatomical evidents.  |
| 1992-1996 | B. Sc. in botany, Ferdowsi University of Mashad, Iran.<br><b>B. Sc. Thesis:</b> Production of non virus plants of <i>Lycopersicum esculentum</i> (Solanaceae) by tissue culture of apical meristem.   |

### ➤ **Carrier**

- |            |  |
|------------|--|
| Since 2002 | Academic member of University of Ferdowsi, Mashad/ Iran. |
|------------|--|

### ➤ **Projects**

- |           |  |
|-----------|--|
| 2002-2005 | Study of morphological characters of Anthemideae, Astereae and Ranunculaceae in Herbarium of Ferdowsi University of Mashad |
|-----------|--|

(FUMH).

2004- 2006 Study of plant floristics and ecology of Fereizi (NE of Iran).

#### ➤ Field work

03-04 2007 Elburz & Zagros Mountains in Iran

07-08 2008 Himalaya Mountains in Nepal

#### ➤ Teaching

2000-2002 General Botany and Systematic of Botany (University of Payame Nour, Iran)

2009 DNA markers in plant systematic and evolution (tutor at the University of Vienna)

#### ➤ Grants & Awards

2006-2009 **Doctoral Scholarship**, Austrian exchange service (ÖAD)  
33,840.00 €

2007 **Best project award (in botany)**  
Ferdowsi University of Mashad, Iran

2008 **Attendance of the conference** (Göttingen, Germany)  
KWA –Konferenzteilnahme, University of Vienna.  
350.00 €

2008 **Attendance of the conference** (Barcelona, Spain)  
KWA- Konferenzteilnahme, University of Vienna.  
350.00 €

2009 **Student bursary**. Attendance of the conference Systematic 2009 (Leiden, Netherland).  
700.00 €

#### ➤ Memberships in scientific societies

Since 2006 International Association for Plant Taxonomy (IAPT)

#### ➤ Publications

- **Published Papers**

**Emadzade, K., & Joharchi, M. R.** 2004. New record, *Psychrogeton cabulicus* (Asteraceae) from Iran. *The Iranian Journal of Botany*, 10: 181-183.

**Emadzade, K., & Joharchi, M. R.** 2005. "Study of Anthemideae (Asteraceae) in Khorassan province on the base of morphological data." *Quarterly Journal of Science, Teacher Training University*, 4: 439-470.

Memariani, F., Joharchi, M.R., Ejtehad, H. & **Emadzade, K.** 2009. A contribution to the flora and vegetation of Binalood mountain range, NE Iran: Floristic and chorological studi in Fereizi region. *Ferdowsi University International Journal of Biological Science*, 1, 1-18.

Hörandl, E., Greilhuber, J., Klimova, K., Paun, O., Temsch, E., **Emadzade, K., & Hodálová, I.** 2009. Reticulate evolution and taxonomic concepts in the *Ranunculus auricomus* complex (Ranunculaceae): insights from morphological, karyological and molecular data. *Taxon* 58: 1194-1215.

- **Manuscripts and book chapters in press**

**Emadzade, K.,** Lehnbech, C., Lockhart P., and Hörandl, E.: A molecular phylogeny, morphology and classification of genera of Ranunculeae (Ranunculaceae). *Taxon*

**Emadzade, K.** Anthemideae. In: *Flora of Khorassan* (Iran)

**Emadzade, K.** Astereae. In: *Flora of Khorassan* (Iran)

**Emadzade, K.** Ranunculaceae. In: *Flora of Khorassan* (Iran)

- **Manuscripts in review**

**Emadzade, K., & Hörandl, E.** (in review) Northern Hemisphere origin, transoceanic dispersal, and diversification of Ranunculeae (Ranunculaceae) in the Tertiary. Submitted to *Journal of biogeography*.

**Emadzade, K.,** Gehrke, B., Linder, P., & Hörandl, E. (in review) The biogeographical history of the cosmopolitan genus *Ranunculus* L. (Ranunculaceae) in the temperate to meridional zones. Submitted to *Molecular phylogenetics and evolution*.

- **Abstract of contributions to conferences/ symposia**

Ghorashi Al-Hosseini, J., & **Emadzade, K.** 2000 “Numerical taxonomic study of Anthemis L. in east of Iran.” In Proceeding of 9th Iranian Biology Conference, p.53, 15-17 Aug.2006. Tehran, Iran.

Ghorashi Al-Hosseini, J., & **Emadzade, K.** 2003 “The systematic study of two genera, Anthemis and Matricaria (Asteraceae) in east of Iran.” In Proceeding of 1<sup>st</sup> International Meeting of Asteraceae, p. 17, 9-10 January 2003. Pretoria, South Africa.

**Emadzade, K.** 2003 “An anatomical study of six species of the genus Anthemis in east of Iran.” In Proceeding of 11<sup>th</sup> Iranian Biology Conference, p.355, 23-25 Aug. 2003, Urmia, Iran.

**Emadzade, K.** 2004 “Production of non virus plants of Lycopersicum esculentum by tissue culture of apical meristem.” In Proceeding of the 2nd Congress on Applied Biology, International Approach, p.136, 29-30 Sep. 2004, Mashad, Iran.

**Emadzade, K.**, Emami-nouri, A. 2005 “A taxonomic study on the tribe of Astereae (Asteraceae) in east & northeast of Iran.” In Proceeding of 17<sup>th</sup> International Congress of Botany, p. 408, 18-23 July 2005. Vienna, Austria.

**Emadzade, K.** 2005 “A taxonomic study on the tribe of Anthemideae in Khorassan province on the base of morphological characters” In Proceeding of 13<sup>th</sup> Iranian Biology Conference, 23-25 Aug., Rasht, Iran.

**Emadzade, K.**, & Joharchi, M. R. 2006 “A revision on the Anthemideae (Asteraceae) in North and Northeast Iran.” In Proceeding of 4<sup>th</sup> International Meeting of Asteraceae, pp. 118, 3-9 July. Barcelona, Spain.

Ejtehadi, A., **Emadzade, K.**, Joharchi, M., Memariani, F. 2006 “Plant diversity of Fereizi region in Binalood mountains, NE Iran.” In Proceeding of 4th Balkan Botanical Congress, p. 216, 20-26 June. Sofia, Bulgaria.

**Emadzade, K.**, Hörandl, E. 2007. Phylogenetic relationships, biogeography and

morphological adaptations of *Ranunculus* in southern Eurasia. In: 6th Biennial Conference of the Systematics Association at the Royal Botanic Gardens Edinburgh, 28-31 Aug., Edinburgh, England.

**Emadzade, K.**, Hörandl, E., C. Lehnebach, & P. Lockhart. 2008 Molecular phylogeny, biogeographical history and a revised classification of *Ranunculus* s.l. (Ranunculaceae). In: 10<sup>th</sup> Annual Meeting of the Gesellschaft für Biologische Systematik & 18<sup>th</sup> International Symposium “ Biodiversity and Evolutionary Biology” of the German Botanical Society, p. 55, 7 -11 April 2008, Göttingen, Germany.

**Emadzade, K.**, Hörandl, E. 2008 Phylogenetic Relationships, Evolution and Biogeography of *Ranunculus* (Ranunculaceae) in the Northern Hemisphere. In: Annual Meeting of the Society for Molecular Biology and Evolution. 5-8 June, Barcelona.

**Emadzade, K.**, & Hörandl, E. 2008 Phylogeny and biogeographical studies of alpine species of *Ranunculus* s.l. (Ranunculaceae) in Eurasia. In: 10<sup>th</sup> Symposium of the International Organization of Plant Biosystematics, p. 9, 2-4 July, Slovakia.

**Emadzade, K.**, & Hörandl, E. 2009 Molecular phylogeny Evolution and biogeography of *Ranunculus* s.l. (Ranunculaceae). In: 1<sup>st</sup> meeting of Biosyst EU, p. 47, 10-14 Aug., Leiden, the Netherland.