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Phylogenetic relationships in *Ranunculus* species (Ranunculaceae) based on nrDNA ITS and cpDNA *trnL-F* sequences

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The genus *Ranunculus* L., with a worldwide distribution, is the largest member of the Ranunculaceae. Here, nuclear ribosomal internal transcribed spacer (ITS) sequence data and chloroplast *trnL-F* sequence data were used to analyze phylogenetic relationships among members of the annual and perennial (Group *Praemorsa*, Group *Rhizomatosa*, Group *Grumosa* and Group non-*Grumosa*) species of *Ranunculus* in Iran. In the strict consensus tree of nrDNA ITS sequence analyses, seven sub-clades were described, based on morphological, karyological, palynological, and ecological features. Within each clade, there were species belonging to more than one Group, and species of a single Group may fall into different clades, revealing that a classification based on underground system characters does not show natural interspecific relationships and must be revised. This is also apparent from *trnL-F* sequence analyses. Based on our results, the *Praemorsa* and *Rhizomatosa* Groups can be merged. The ITS sequence data show interspecific relationships more clearly than *trnL-F* sequence data. © 2011 Progress in Biological Sciences, Vol. 1, No.1, 41-47.

KEY WORDS: *Ranunculus*; ITS; *trnL-F*; phylogeny; Iran

INTRODUCTION

Ranunculus L. (buttercup) is the largest genus of Ranunculaceae, comprising c. 600 species (Tamura, 1995) with an almost cosmopolitan distribution. Most species occur in temperate to arctic/sub-Antarctic zones, rarely in the tropics, where they are mainly restricted to high elevation areas. With about 55 species, including 19 endemic, the genus has one of its diversification centers in Iran (Iranshahr et al., 1992). *Ranunculus* grows in a wide variety of habitats including forests, dry and damp meadows, wet soils, lakes, rivers, and alpine heaths. They are herbaceous; annual or, more often, perennial; with compound or entire leaves (Johansson, 1998). Various morphological adaptations and reproductive strategies such as vegetative reproduction (stolons), self compatibility (in water-buttercups), and agamospermy (*R. auricomus* complex) may be important factors in their ability to colonize different habitats, altitudes, and latitudes (Hörandl et al., 2005).

In the taxonomic history of the genus, Tamura's treatment (1993, 1995) represents the most recent worldwide revision. The subgeneric classification in these studies is based mainly on achene characters. Seven subgenera have been considered in the genus, i.e. *Pallasiantha*, *Coptidium*, *Ficaria*, *Batrachium*, *Crymodes*, *Gampsoceras,* and *Ranunculus* (Tamura, 1993, 1995). The subgenus *Ranunculus* in turn is subdivided into 20 sections. The chief differences among taxonomic treatments of the genus (Ovczinnikov, 1937; Davis, 1965; Iranshahr et al., 1992) involve uncertainty with respect to intra-generic relationships, i.e. the boundaries between sections and subgenera.

According to Iranshahr et al. (1992) *Ranunculus* should be divided into three groups, annual species, perennial species with grumosa roots

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Table 1. Species of *Ranunculus* used in ITS and *trnL-F* study.

Species	Collection Site	Collector	Herbarium number
R. amblyolobus Boiss. & Hohen.	Damavand, TAR lake(Havir)	Rastipisheh	ALUH3028
R. polyanthemos L.	Damavand, TAR lake(Havir)	Rastipisheh	ALUH3014
R. brutius Ten.	Gilan, Rudbar	Leg. P. Wendlebo, Annala	TARI18174
R. repens L.	Mazandaran, Ramsar, Javaherdeh	Rastipisheh	ALUH3013
R. caucasicus M. B.	Azarbayejan, Arasbaran	Pakravan	ALUH3031
R. constantinopolitanus (DC.) d'Urv	Mazandaran, Ramsar, Javaherdeh	Pakravan	ALUH2075
R. kotschyi Boiss.	Damavand, TAR lake(Havir)	Rastipisheh	ALUH3010
R. ophioglossifolius Vill.	Sari, Soote village	Pakravan	ALUH3654
R. sericeus Banks & Soland.	Saveh	Mozafariyan, Masoomi	TARI48180
R. sojakii Iranshahr. & Rech. f.	Tehran, Dizin	Rastipisheh	ALUH3011
R. arvensis L.	Kashan, Niyasar	Rastipisheh	ALUH3012

(claw-like tubers), and perennial species without grumosa roots.

There are few molecular phylogenetic studies of *Ranunculus*, among which the most important utilized cpDNA restriction sites (Johansson, 1998), cpDNA *matK/trnK* sequences of mainly European species (Paun et al., 2005), and nrDNA ITS sequences (Hörandl et al., 2005). Recently Emadzadeh et al. (2010) reported an inclusive phylogenetic analysis based on a combined dataset of selected nuclear and chloroplast markers. All published phylogenies of *Ranunculus* show considerable inconsistencies with previous taxonomic classifications (for example with Tamura, 1995). On the sectional level, hybridization has probably led to reticulate relationships in many groups (Hörandl et al., 2005).

In this study we used sequence data of nrDNA ITS and cpDNA *trnL-F* to investigate interspecific relationships within *Ranunculus* in Iran and to develop a natural classification.

MATERIAL AND METHODS

Taxon sampling

Twenty-seven accessions representing the 23 species of known sections and sub-genera of *Ranunculus* growing in Iran were used for nrDNA ITS sequences. Eighteen accessions were obtained from GenBank (Tables 1, 2). We used two accessions for each of the species *R. arvensis*, *R. repens*, *R. caucasicus*, and *R. polyanthemos* one of which was obtained from GenBank. The taxon sampling represented seven species of annuals, nine species of *Praemorsa* Group, two species of *Rhizomatosa* Group, two species of *Grumosa* Group and three species of non-*Grumosa* (folia indivisa) Group (Iranshahr et al., 1992). For *trnL-F* sequences, 12 accessions representing 12 species of *Ranunculus* were used, two of which were taken from GenBank (Tables 1, 2). The selected species represented two species of annuals, seven

Table 2. GenBank number of species used in ITS and *trnL-F*.

Species	ITS nrDNA	*trnL-F* cpDNA
R. bulbosus L.	AM503891	FJ490812
R. muricatus L.	DQ410718	DQ410740
R. arvensis L.	AY680177	AB617672
R. caucasicus M.B.	AY680178	AB617674
R. amblyolobus Boiss. & Hohen	AB617666	AB617671
R. polyanthemos L.	AY680121	AB617679
R. kotschyi Boiss	AB617669	AB617676
R. sojakii Iranshahr. & Rech.f.	AB617670	AB617678
R. constantinopolitanus (DC.) d'Urv	AB617668	AB617675
R. repens L.	AY680160	AB617680
R. brutius Ten.	AB617667	AB617673
R. ophioglossifolius Vill.	AY680180	
R. sericeus Banks & Soland.	-	AB617677
R. hirtellus Royle.	AY680038	-
R. marginatus Urv.	AY680190	-
R. cornutus DC.	AY680153	-
R. cicutarius Schlecht.	AY680103	-
R. illyricus L.	AY680119	-
R. rufosepalus Franch.	AY680047	-
R. longicaulis C. A. Mey.	AY680051	-
R. lingua L.	AY680184	-
R. pseudohirculus Schrenk ex F. E .L. Fischer & C. A. Mey.	AY680111	-
R. chius DC.	AY680176	-
R. lateriflorus DC.	AY680179	-

species of the *Praemorsa* Group, and three species of the *Rhizomatosa* Group.

In both analyses, *R. brutius* was selected as an outgroup, based on the molecular study published by Hörandl et al. (2005).

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number(s), AB617666, AB617667, AB617668, AB617669, AB617670, AB617671, AB617672, AB617673, AB617674, AB617675, AB617676, AB617677, AB617678, AB617679, AB617680.

DNA extraction, amplification and sequencing

DNA was extracted from 1-2 g of dried leaf material (silica gel dried or herbarium specimens) using a modified CTAB protocol (Doyle and Doyle, 1987). For amplification and sequencing of the ITS region, the forward (ITS5) and reverse (ITS4) primers of White et al. (1990) were used (Table 3). The *trnL-F* spacer was amplified and sequenced using primers *trn-c* and *trn-f* of Taberlet et al. (1991) (Table 3). PCR for the ITS region was carried out in 50 µl reactions using 5 µl DNA, 2 µl of each primer, 3 µl dNTP (concentration 2.5 mM), 5 µl Buffer (+ Mg^{++}), 0.2 µl Taq Polymerase (1 U/µl), and 33.8 µl water. Reaction conditions for the ITS region were: denaturation at 94°C for 3 min followed by 30 cycles of 1 min at 94°C, 45 sec at 51°C, 2 min at 72°C, and a final extension at 72°C for 10 min in a Peltier thermal cycler (PTC 200; MJ Research). PCR for *trnL-F* region, was carried out in 52 µl reactions using 3 µl DNA, 1 µl of each primer, 5 µl dNTP (concentration 2.5 mM), 5 µl Buffer, 0.5 µl Taq Polymerase (concentration 1 U/µl) and 36 µl water. PCR conditions for the *trnL-F* region were: denaturation at 94°C for 4 min followed by 30 cycles of 1 min at 94°C, 45 sec at 64°C, 2 min at 72°C and a final extension at 72°C for 5 min in a Peltier thermal cycler (PTC 200; MJ Research). Amplified PCR products were purified using QIAquick PCR Purification kits (QIAGEN, Hilden, Germany) and sequenced using an ABI Big-Dye Ready Reaction kit with an ABI 3730xl DNA Analyzer 96 capillary automated sequencer.

Table 3. Sequences of the primers used for amplification of the ITS region and *trnL-F* spacer

Primer Name	Sequence (5'- 3')
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG
trn-c	CGAAATCGGTAGACGCTACG
trn-f	ATTTGAACTGGTGACACGAG

Phylogenetic analyses

Verified sequences were aligned by sight using Bioedit (version 5.0.6). Maximum parsimony (MP) analyses were performed using PAUP* version 4.b10 (Swofford, 2003) with the heuristic search options. For this purpose, heuristic searches were performed with 1,000 random

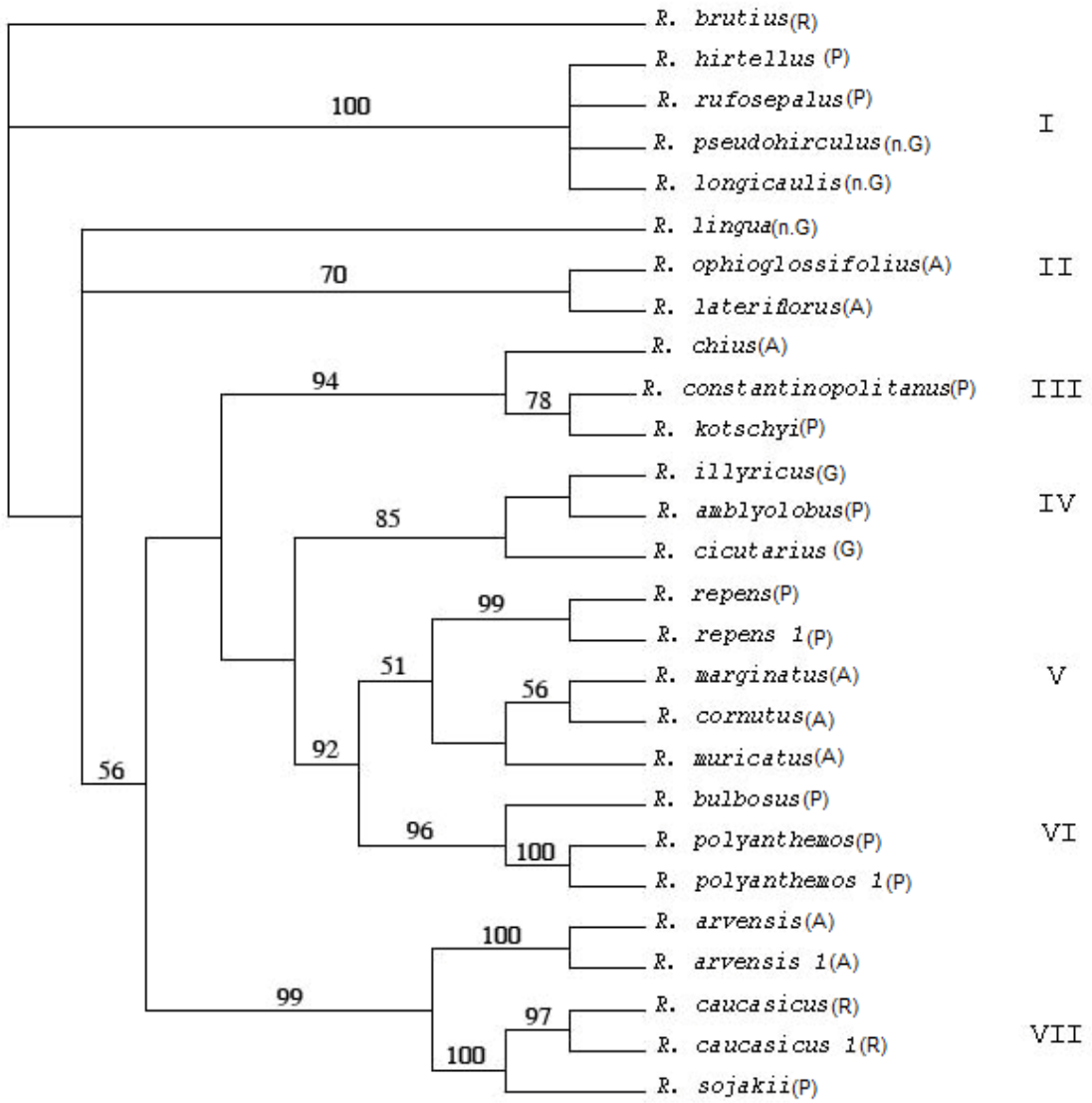

Fig. 1. Strict consensus of 29 trees resulting from parsimony analysis of ITS data. Bootstrap values larger than 50 are indicated above the corresponding branches. R (*Rhizomatosa*), P (*Praemorsa*), G (*Grumosa*), n. G (non-*Grumosa*), A (annual species).

stepwise addition replicates and TBR branch swapping with the MULTREES option in effect. The strict consensus tree was computed from all equally most parsimonious trees. The internal support for individual branches was estimated using nonparametric bootstrapping (Felsenstein, 1985). Bootstrap values are shown on the corresponding clades of the strict consensus tree of the parsimony analysis. In addition, a neighbor-joining analysis for each of the datasets was conducted. The results of the analyses show the same topology as the parsimony analyses.

RESULTS

The aligned data matrix of nrDNA ITS sequences was 731 bp. Five ITS sequences were reported for the first time (*R. amblyolobus*, *R. sojakii*, *R. kotschyi*, *R. brutius*, and *R. constantinopolitanus*). The heuristic search yielded 29 most parsimonious trees with length (L) of 494 steps, CI of 0.7753, and RI of 0.7849. The strict consensus of 29 trees with the corresponding bootstrap values is shown in Fig. 1. The cladogram obtained includes a set of well-supported clades designated I-VII.

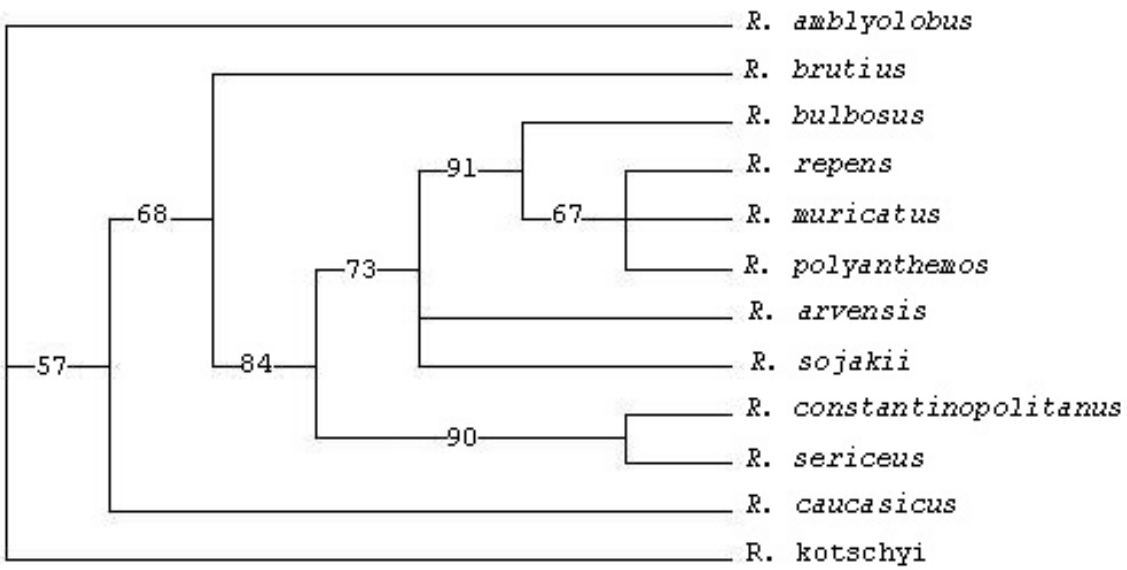

Fig. 2. The strict consensus of 6 trees resulting from parsimony analysis of *trnL-F* data. Bootstrap values >50 are indicated above the corresponding branches

The aligned data matrix of *trnL-F* sequences was 478 bp after removing ambiguous positions at the end of the matrix. Ten species were sequenced for the first time for this intergenic spacer (*R. amblyolobus*, *R. arvensis*, *R. sojakii*, *R. caucasicus*, *R. kotschyi*, *R. brutius*, *R. constantinopolitanus*, *R. sericeus*, *R. polyanthemos*, and *R. repens*). The heuristic search yielded six most parsimonious trees with L=459 steps, CI=0.9107, and RI=0.6059. The strict consensus of these six trees is shown in Fig. 2.

DISCUSSION

The nrDNA ITS phylogeny study conducted here shows a high level of congruence with the phylogeny reconstruction by Hörandl et al. (2005) and Paun et al. (2005). Our results, however, contradict previous classifications of the genus based only on morphological characters (Davis, 1965; Iranshahr et al., 1992; Tamura, 1995). The molecular data yielded a set of well-supported clades (I-VII) (Fig. 1), each clade representing a collection of species with common characters.

Clade I

Clade I is well-supported and includes *R. hirtellus* (*Praemorsa* Group), *R. rufosepalus* (*Praemorsa* Group), *R. pseudohirculus* (non-*Grumosa* Group, folia individa) and *R. longicaulis* (non-*Grumosa* Group, folia individa). This clade corresponds to the classifications of Tamura (1995). These species are nested in the subgenus *Ranunculus* (sect. *Ranunculus*). The species of this clade show primarily a Central Asiatic distribution, which indicate also their affinity.

Clade II

Clade II includes *R. lingua* (sect. *Flammula*), *R. ophioglossifolius* (sect. *Flammula*) and *R. laterifolius* (sect. *Micranthus*). *R. lingua* is sister to *R. ophioglossifolius* and *R. laterifolius*, but, palynologically (see Clarke et al., 1991) is placed in a separate group (*Lingua* group). These three species are placed in two sections (*Flammula* and *Micranthus*) based on Tamura's classifications. The two sections share some characteristics, such as undivided leaves, more or less swollen achenes, and small beaks. In addition,

they show a similar karyotype (D'Ovidio and Marchi, 1990).

Clade III

R. chius, *R. constantinopolitanus*, and *R. kotschyi* are also nested in a well supported clade III (bp = 94%). Within this clade, the basic chromosome number is x = 7. This clade can be defined by compressed and bordered achenes, terete pedicels, erect sepals, and glabrous receptacles (Hörandl et al., 2005). *Ranunculus constantinopolitanus* and *R. kotschyi* have many similar characteristics, differing only in the shape of achene and density of hairs.

Clade IV

R. illyricus, *R. amblyolobous*, and *R. cicutarius* are nested in clade IV. *Ranunculus illyricus* and *R. cicutarius* are classified in the *Grumosa* Group, while *R. amblyolobous* is a member of the *Praemorsa* Group. The latter shares few palynological characters with *R. cicutarius*. Both species are placed in the *R. acris* group. Pollen grains of *R. acris* group are easily recognizable by an irregular, coarsely undulating tectum. Most species of this group have pollen grains with more than three colpi (up to 12) which are randomly arranged over the surface (pantocolpate). These colpi often have irregular, indistinct margins (Clarke et al., 1991).

Clade V and VI

Within clade V, *R. repens* is sister to the annual species *R. marginatus*, *R. curnatus*, and *R. muricatus*. These are wetland plants and distributed in similar habitats. *Ranunculus repens* is morphologically and palynologicaly similar to the species of clade VI, *R. polyanthemos* and *R. bulbosus*. *Ranunculus repens*, *R. polyanthemos*, and *R. bulbosus* are nested within the *Praemorsa* Group and share morphological characters.

Clade VII

This clade includes *R. arvensis*, *R. caucasicus*, and *R. sojakii*. *Ranunculus arvensis* has the typical flat spiny fruit with a xylem anatomy peculiar in having a pinnate venation of the pericarp with several ramifications (Trzaski, 1999). The pollen grains in this species are perporate and have echinate tectum. These features are not found in other annual species. It forms a sister group to *R. caucasicus* and *R. sojakii* which are perennials. *R. caucasicus* and *R. sojakii* resemble the species of clade V and VII In morphology.

The resultant tree of the *trnL-F* region is not congruent with the ITS tree, and the relationships of branches to each other are unresolved, with the exception of *R. constantinopolitanus* of the *Praemorsa* Group and *R. sericeus* of the *Rhizomatosa* Group, which form a strongly supported clade (90% bootstrap). These species are morphologically similar to each other.

The categorization of *R. bulbosus*, *R. repens*, *R. polyanthemos*, *R. muricatus*, *R. arvensis*, and *R. sojakii* in one sub-clade in the *trnL-F* tree (Fig. 2) is consistent with the results of ITS tree. As *trnL-F* sequences provide a low number of informative characters, and, due to ambiguous positions among these sequences, we do not suggest the use of such non-coding regions of the chloroplast genome in low level taxonomy of *Ranunculus*. Therefore, the results of *trnL-F* sequences are not discussed further.

Our data show that the *Rhizomatosa* and *Praemorsa* Group can be merged. In addition, the results presented here demonstrate that the classification of perennial species based on the underground system is not supported, and the type of root is an adaptive character related to climate and altitude.

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REFERENCES

Clarke GCS, Punt W, Hoen PP (1991) Ranunculaceae. In The northwest European pollen flora VI (Blackwell:London, UK), PP.117-271

Davis PH (1965) Material for a flora of Turkey, Ranunculaceae II. Ranunculus. Notes Roy Bot G Edinb 23, 103-161

D'Ovidio R, Marchi P (1990) DNA content, karyotype structure analysis and karyotype symmetry in *Ranunculus* L (Ranunculaceae). Italian species belonging to sections Flammula (Webb) Benson and Micranthus (Ovcz) Nyarady. Caryologia 43, 99-115

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19, 11-15

Emadzade Kh, Lehnebach C, Lockhart P, Horandl E (2010) A molecular phylogeny, morphology and classification of genera of Ranunculeae (Ranunculaceae). Taxon 59, 809-828

Felsenstein J (1985) Confidence limits on phylogenetics: an approach using the bootstrap. Evolution 39, 783-791

Horandl E, Paun O, Johanson JT, Lehnebach C, Armstrong T, Chen L, Lockhart P (2005) Phylogenetic relationships and evolutionary traits in *Ranunculus* s.l. (Ranunculaceae) inferred from ITS sequence analysis. Mol Phyl Evol 36, 305-327

Johansson JT, Jansen RK (1993) Chloroplast DNA variation and phylogeny of the Ranunculaceae . Pl Syst Evol 187, 29-49

Johansson JT (1998) Chloroplast DNA restriction site mapping and the phylogeny of *Ranunculus* (Ranunculaceae). Pl Syst Evol 213, 1-19

Ovczinnikov PN (1937) Flora URSS, Vol 2, Ranunles and Rhoeadales, (Akademi Nauk URSS, Moscow) (English translated).

Paun O, Lehnebach C, Jahonsson JT, Lockart P, Horandl E (2005) Phylogeny relationship and biogeography of *Ranunculus* and allied genera (Ranunculaceae) in the Mediterranean region and in the European alpine system. Taxon 54, 911-930

Rechinger KH, ed. (1992) Ranunculaceae. Flora Iranica. (Graz: Akad. Druck- u. Verlagsanstalt) 171, 1–249

Swofford DL (2003) Sinauer Associates, Sunderland, Massachusettes. PAUP*: Phylogenetic Analysis Using Parsimony. Version 4.b10.

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non_coding regions of chloroplast DNA. PL Mol Bio 17, 1105-1109

Tamura M (1993) Ranunculaceae. in: The families and genera of vascular plants. 2. flowering plants, Dicotyledons (Magnoliid, Hamamelid, Caryophyllid families) II, Kubitzki K, Rohwer JG, Bittrich, V, eds. (Springer: Berlin)

Tamura M (1995) Angiospermae odnung Ranunculales Fam. In: Phylogeny relationship and biogeography of Ranunculus and allied genera (Ranunculaceae) in the Mediterranean region and in the European alpine system, Paun O, Lehnebach C, Jahonsson JT, Lockart P, Horandl E . Taxon 54, 911-930

Trzaski L (1999) Xylem distribution in the achene of some European *Ranunculus* species as a taxonomical criterion of *Ranunculus* genus. Phytomorph 49, 241-251

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fung al ribosomal RNA genes for phylogenetics. In: PCR Protocols: A guide to methods and applications, Innis M, Gelfand D, Sininsky J, White T, eds. (Academic Press, Inc: New York, USA), pp. 315-322