

Phylogeny of *Hepatica* (Ranunculaceae) and origin of *Hepatica maxima* NAKAI endemic to Ullung Island, Korea

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Abstract: Nuclear ITS, chloroplast *atpB-rbcL* spacer and *trnL-F* intron and spacer sequences, as well as amplified fragment length polymorphism (AFLP) data, have been analyzed to infer a molecular phylogeny for the genus *Hepatica* and to determine the presumptive progenitor species of the Ullung Island (Korea) endemic *H. maxima*. Although all DNA sequence markers clearly demonstrate the monophyly of *Hepatica* and a sister group relationship with species of *Anemone* that share the same basic chromosome number (x = 7), they fail to reveal relationships within *Hepatica*. However, six synapomorphic indels and four mononucleotide repeats (microsatellites) have been detected in the plastid *trnL-F* sequences among species of the genus. Based on these data, at least 14 plastid types can be discriminated among populations of *H. asiatica*, *H. henryi*, *H. insularis*, and *H. maxima*. Both *H. asiatica* and *H. insularis* populations consist of various plastid haplotypes that do not cluster following specific distinctions based on morphology. The data suggest that *H. maxima* has originated from populations of *H. asiatica* located in the Kangwon Province of eastern South Korea. AFLP data confirm clustering of *H. maxima* populations with those of *H. asiatica* from peninsular Korea and not with any populations of taxa from Japan (vars. of *H. nobilis*). No genetic separation between *H. asiatica* and *H. insularis* populations was detected by nuclear AFLP markers; the two species appear to be genetically closely related. The various plastid types may have resulted from lineage sorting of uniparentally inherited plastids.

Zusammenfassung: Nukleäre ITS, plastidäre *atpB-rbcL*-IGS und *trnL-F*-Intron und IGS-Sequenzen, sowie amplified fragment length polymorphism (AFLP) Daten wurden analysiert um eine molekulare Phylogenie der Gattung *Hepatica* zu erstellen und um die Abstammung von *H. maxima*, einem Endemiten der korenischen Insel Ullung Island, aufzuklären. Obwohl alle DNA-Sequenz-Marker eindeutig die Monophylie der Gattung *Hepatica* bestätigen und die phylogenetische Verwandtschaft zu den Arten der Gattung *Anemone* mit der gleichen basalen Chromosomenanzahl wie *Hepatica* (x = 7) belegen, erlauben sie keine Differenzierung innerhalb der Gattung *Hepatica*. Allerdings wurden in den plastidären *trnL-F* Sequenzen sechs synapomorphe Indels und vier Mononukleotid-Repeats gefunden. Aufgrund dieser Daten war es möglich mindestens 14 verschiedene Plastidentypen in Populationen von *H. asiatica*, *H. henryi*, *H. insularis* und *H. maxima* zu bestimmen. Populationen von *H. asiatica* und *H. insularis* bestehen aus verschiedenen plastidären Haplotypen, die allerdings nicht mit der morphologischen Zuordnung zu den einzelnen Arten korrelieren. Die Daten lassen eine Abstammung des Endemiten *H. maxima* von *H. asiatica*-Populationen aus der Provinz Kangwon im östlichen Teil Südkoreas vermuten. AFLP-Daten bestätigen die nahe Verwandtschaft von *H. maxima*-Populationen mit *H. asiatica*-Populationen vom koreanischen Festland und widerlegen eine nähere Verwandtschaft zu japanischen Populationen (Varietäten von *H. nobilis*). Keine genetische Differenzierung zwischen *H. asiatica* und *H. insularis*-Populationen konnte aufgrund der nukleären AFLP-Daten detektiert werden; offensichtlich sind beide Arten nahe verwandt und kaum genetisch differenziert. Die unterschiedlichen Plastiden-Haplotypen stellen deshalb möglicherweise das Resultat von Lineage-Sorting von uniparental vererbten Plastiden dar.

Key words: AFLP analysis, *Hepatica*, island evolution, nuclear ITS sequences, plastid DNA sequences, plastid microsatellites, speciation.

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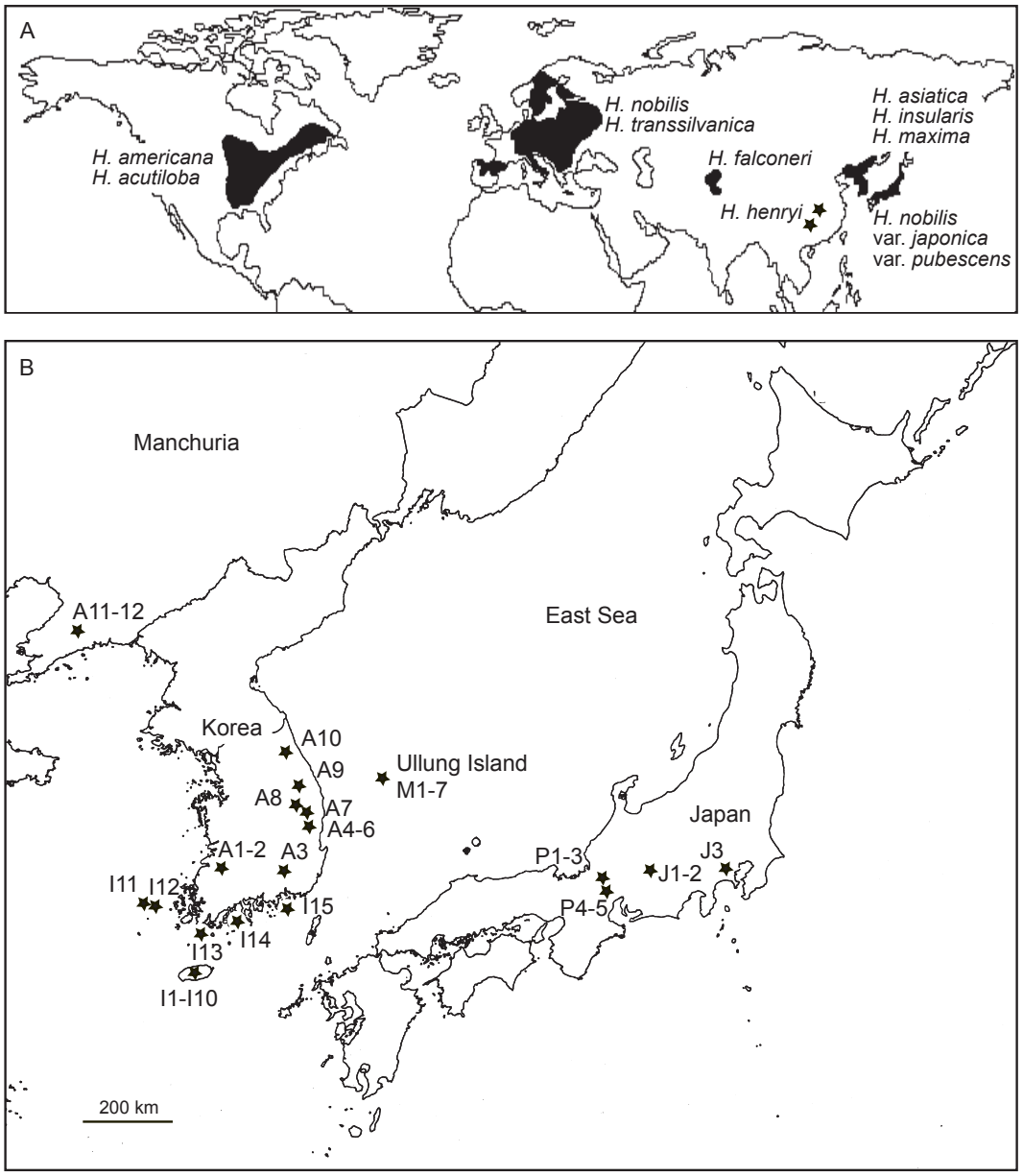

Fig. 1: A, Geographical distribution of *Hepatica*. B, Location of Ullung Island in the Eastern Sea and localities of sampled populations of *H. asiatica* (A1-12), *H. insularis* (I1-15), *H. maxima* (M1-7), *H. nobilis* var. *japonica* (J1-3), and *H. nobilis* var. *pubescens* (P1-5).

Introduction

Islands are attractive systems for studying plant evolution for a number of reasons: they present discrete geographical entities within defined oceanic boundaries, gene flow is reduced by oceanic barriers, and despite their small geographical size, they may host a diversity of habitats and often contain high levels of endemism (Emerson 2002; Grant 1998; Stuessy & Ono 1998; Whittaker 1998). Such endemic plants have been suggested as model systems for the study of plant speciation (Crawford & Stuessy 1997). Patterns and processes of plant evolution are often more clearly visible in island endemics because gene flow and genetic exchange are reduced by their geographical isolation from source areas. Recent years have seen an increasing number of investigations using molecular data on various evolutionary phenomena in different island systems, including the Hawaiian Islands (Baldwin & Sanderson 1998; Barrier et al. 1999), Juan Fernandez Islands (Stuessy & Ono 1998), Canary Islands (Francisco-Ortega et al. 2001; Ray 1995), and Macaronesia (Francisco-Ortega et al. 1999; Vargas et al. 1999). One of the most recent additions to island systems in which molecular investigations of selected endemic taxa have been completed is the Korean island Ullung-do (Pfosser et al. 2002). This system has the experimental advantage that all examples for plant speciation on this island appear to be the result of simple anagenetic changes, without complications from subsequent intra-island cladogenetic speciation events (Sun & Stuessy 1998).

The small oceanic island Ullung-do is located 137 km east of mainland Korea and c. 300 km west from Japan (Fig. 1), extending from 37°27′ to 37°33′ North and from 130°47′ to 130°56′ East. The total area of the island is 73 km² and the highest peak in the center of the island is 984 m above sea level. The island is of volcanic origin and no connections to other landmasses ever existed (KIM 1985a, b). Potassium-argon dating methods revealed first volcanic eruptions under the sea about 2.7 million years ago, and the maximum age of the island during which colonization by diaspores was possible has been determined to be 1.8 million years (KIM 1985a). A recent controversial hypothesis, however, postulates a late Tertiary origin from three to five million years for Ullung-do, and even suggests direct land connections to the Korean peninsula and the west coast of Japan until the early Quaternary (YANG 1996). This hypothesis seems unlikely because of deep water on both sides of Ullung Island and the absence of ridges or seamounts that could have connected it to Japan or the Korean peninsula.

Among the c. 700 vascular plant species on Ullung Island are 37 endemic angiosperm taxa, representing 25 families and 34 genera (LEE & YANG 1981; SUN & STUESSY 1998). Closest floristic relationship has been determined between Ullung Island and Korea (94% of species in common), Japan (86%) and Manchuria (59%; (KIM 1988)). Focus for the molecular studies has been on revealing the evolutionary origin of these endemic species. The origin of the two endemic species of *Acer*, *A. okamotoanum* and *A. takesimense*, has recently been shown (PFOSSER et al. 2002) to derive from progenitors resembling *A. mono* and *A. pseudosieboldianum*, respectively, from the Korean peninsula. Another endemic Ullung Island species, *H. maxima*, is the focus of the present investigation.

The genus *Hepatica* MILLER (Ranunculaceae) comprises six (MELCHIOR 1964), seven (TAMURA 1993), or nine species (WEISS et al. 2002), depending upon taxonomic perspective, and is distributed in temperate environments of Europe, East Asia and central and eastern North America (Fig. 1). The highest diversification is found in northeastern Asia with four species and two varieties including the Ullung endemic *H. maxima*. NAKAI (NAKAI 1937; NAKAI 1952) has divided the Korean species into two groups, *H. asiatica* and *H. insularis* with annual leaves and the Ullung Island endemic, *H. maxima*, with biennial leaves. Two varieties of the European *H. nobilis*, var. *japonica* and var. *pubescens* are found in Japan. The latter variety has been reported as hexaploid (HIROE 1957). Both *H. henryi* from China and *H. transsilvanica* (Transcarpathians) are tetraploids. Additional species occur in North America *(H. acutiloba* and *H. americana;* STEYERMARK & STEYERMARK 1960*)* and the threatened species *H. falconeri* is known from the Kashmir and Pamir regions. The worldwide distribution of *Hepatica*, therefore, shows a disjunct appearance with several localized endemics (JALAS & SUOMINEN 1989). Although *H. maxima* shows dramatic changes from its putative progenitor, morphological analysis, based on leaf characters, suggests that the closest relative is *H. asiatica* from mainland Korea (SUN & STUESSY 1998). Likewise, the second Korean endemic, *H. insularis* from Cheju Island and southern coastal regions of peninsular Korea, appears morphologically closely related to *H. asiatica* (SUN & STUESSY 1998; WOO et al. 2002). Both endemics have been treated sometimes as forms or varieties of *H. asiatica* (LEE 1976; PARK 1974).

No previous phylogenetic investigations among species of *Hepatica* have been completed. A few species have been included in analyses aimed at resolving relationships among species of *Anemone* and related genera (EHRENDORFER et al. 2009; EHRENDORFER & SAMUEL 2001; HOOT et al. 1994; MEYER et al. 2010; SCHUETTPELZ et al. 2002). These analyses convincingly demonstrate that *Hepatica* is closely related to species of *Anemone* that have the same basic chromosome number of $x = 7$, but they are insufficient for determining possible progenitors for the Ullung Island endemic *H. maxima*.

The present investigation is based on analysis of nuclear ITS, plastid *trnL-F* intron and spacer, and *atpB-rbcL* spacer sequences to construct a molecular phylogeny for *Hepatica* worldwide. To increase the resolution of the analysis, plastid microsatellite data have been obtained and analyzed. Amplified fragment length polymorphism (AFLP) data have also been used to investigate population parameters of endemics and putative progenitors.

Materials and Methods

Taxa Sampled. This analysis is based on material of *Hepatica* sampled in peninsular Korea, Ullung-do (Korea), Cheju Island (Korea), China, Austria, U.S.A., and Japan during 1999-2001. Additional material (*H. transsilvanica*, *H. henryi*) has been obtained from Botanical Gardens or private collectors). Voucher information for all plant accessions, geographic origin, and EMBL database accession numbers are provided in the Appendix. Vouchers are deposited in WU unless otherwise indicated.

DNA Extraction. Total genomic DNA was prepared from fresh or silica gel-dried leaf material following the isolation protocol of DOYLE & DOYLE (1987) with slight modifications. The powdered leaf material was extracted in 700 µl CTAB buffer (2% CTAB, 100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 0.2% mercaptoethanol, pH 8.0) for 30 minutes at 60°C. 500 µl chloroform/isoamylalcohol (24/1) were added and the extraction mix was incubated for 15 minutes at 4°C. After centrifugation, the DNA was precipitated with 500 µl isopropanol. The pellet was washed with 70% ethanol and dissolved in 100 µl TE buffer.

DNA Sequencing. Three non-coding regions of the chloroplast genome were sequenced. The **trnL(UAA)** intron and the intergenic spacer (IGS) between the **trnL(UAA)**-3′exon and the **trnF(GAA)** gene were amplified together in a single PCR reaction (PFOSSER & SPETA 1999). The **atpB-rbcL** intergenic spacer was amplified using the primers 2 and 5 of MANEN et al. (1994). Nuclear ITS sequences were amplified with the primers ITS1-1 (5′-cgagaagtccctgaacctta–3′) and ITS4 (5′-cctccgcct-tattgatatg–3′). Amplified double-stranded DNA fragments were sequenced directly on an ABI377 automated sequencer (Perkin Elmer, UK) following the DYEnamicET cycle sequencing protocol (Amersham Pharmacia, USA) or the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, NJ), using either nested sequencing primers (PFOSSER & SPETA 1999) or the primers used for amplification. Two internal sequencing primers ITS2 (5′-gctacgttcttcatcgatgc-3′) and ITS3 (5′-gcatcgatgaagaacgtagc-3′) were used to complete sequencing of the ITS region in both directions. On average, less than 1% of data matrix cells were scored as missing data.

Phylogenetic Analysis. Sequence manipulations were performed on a Digital Alpha 1000A 5/400 server under the operating system

Fig. 2: Majority rule consensus tree based on maximum parsimony analysis of nuclear ITS sequences of *Hepatica* spp. and selected species of the genera *Anemone*, *Clematis*, *Pulsatilla*, and *Knowltonia*. The tree is rooted with the genera *Trautvetteria* and *Ranunculus*. Bootstrap support values (10,000 replicates) higher than 50% are indicated above branches. Nodes collapsing in the strict consensus tree are marked by arrows. The divergence between basal chromosome numbers of $x = 7$ or $x = 8$ among members of Anemoninae is indicated by background shading. The Ullung Island endemic *H. maxima* is shown in bold.

Digital Unix V.4.0D. DNA sequences were pre-aligned using the PileUp program of the GCG software package (Genetics Computer Group 1994) and trimmed on both ends to compensate for different sequence lengths. Final alignment of DNA sequences was done visually. The sequences have been trimmed on both ends to exclude ambiguous positions in close proximity to the sequencing primers. All sequences have been deposited in the EMBL database (for accession numbers refer to Appendix 1). Phylogenetic analysis using the maximum parsimony (MP) or neighbor-joining (NJ) method was performed with the computer program PAUP version 4.0b4 (SWOFFORD 2000). Most parsimonious trees were obtained by 1,000 replicates of random sequence addition using tree bisection-reconnection (TBR) branch swapping under the Fitch criterion (FITCH 1971). Successive character weighting was applied and new heuristic searches were performed using the trees of the previous analysis as starting trees until tree lengths remained the same in two successive rounds. Ten thousand fast bootstrap replicates (FELSENSTEIN 1985) were used to assess confidence limits for the resulting tree topologies. Indels (insertions/deletions) in the *trnL-F* data matrix were coded as additional characters, the

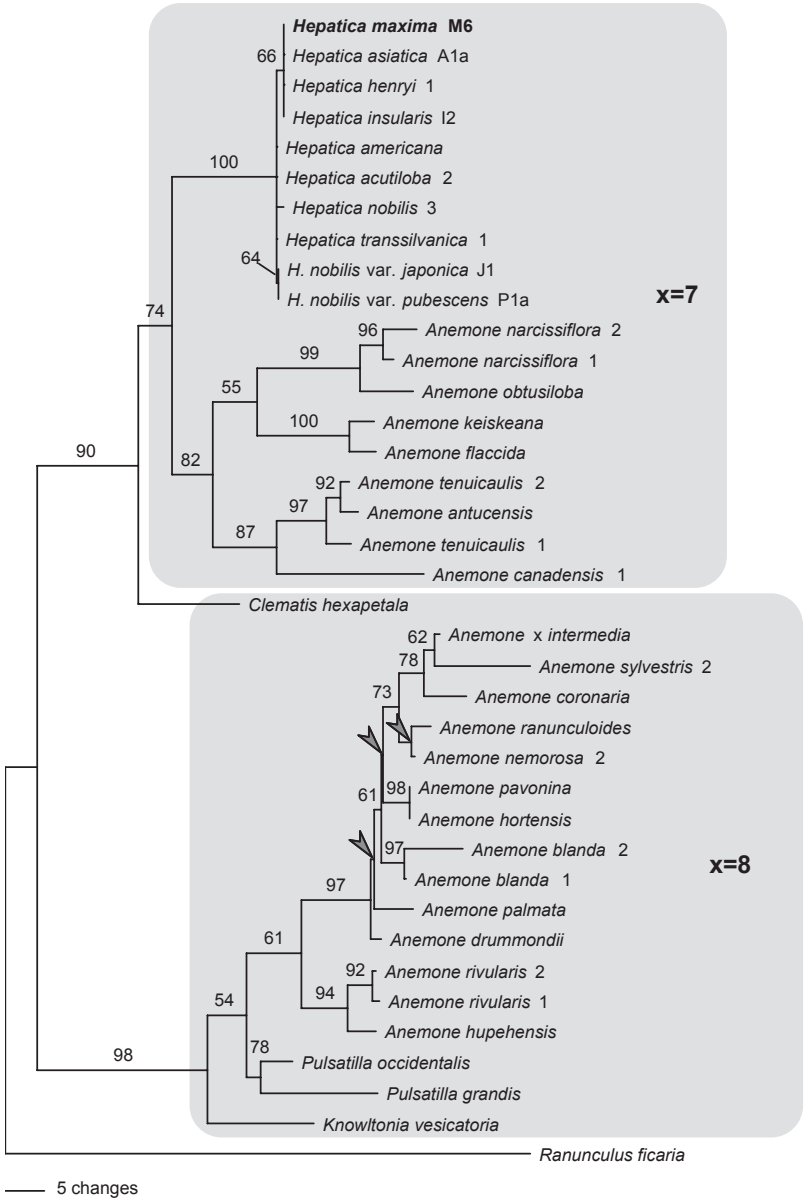

Fig. 3: Majority rule consensus tree based on maximum parsimony analysis of plastid *atpB-rbcL* spacer sequences of *Hepatica* spp. and selected species of the genera *Anemone*, *Clematis*, *Pulsatilla*, and *Knowltonia*. The tree is rooted with *Ranunculus ficaria*. Bootstrap support values (10,000 replicates) higher than 50% are indicated above branches. Nodes collapsing in the strict consensus tree are marked by arrows. The divergence between basal chromosome numbers of $x = 7$ or $x = 8$ among members of Anemoninae is indicated by background shading. The Ullung Island endemic *H. maxima* is shown in bold.

nucleotide stretch corresponding to one gap (or two overlapping indels) being conservatively considered as a single site with several states, regardless of its length. Four plastid microsatellites (poly-T or poly-A runs) were encountered in the *trnL-F* region corresponding to nucleotide positions 119-129, 684-692, 760-771, 1026-1034 of the aligned data matrix. Tree searches were performed using the nucleotide data alone or together with the indel and microsatellite data. Tree manipulations were performed using MacClade version 3.06 (MADDISON & MADDISON 1992).

AFLP Analysis. AFLP analysis was performed according to the protocol provided with a commercial AFLP analysis kit (Life Technologies, UK) with minor modifications. 200 ng DNA were digested for 3 hours with the restriction enzymes **Eco**RI and **Mse**I in a total volume of 5 µl. Adapters (5 µl) were added and restriction and ligation were allowed to continue overnight at 37°C. A 0.5 µl aliquot of the ligated DNA fragments was pre-amplified in a TouchDown thermal cycler (HYBAID) during 20 cycles of 20 sec at 94°C, 40 sec at 56°C, 50 sec at 72°C. The

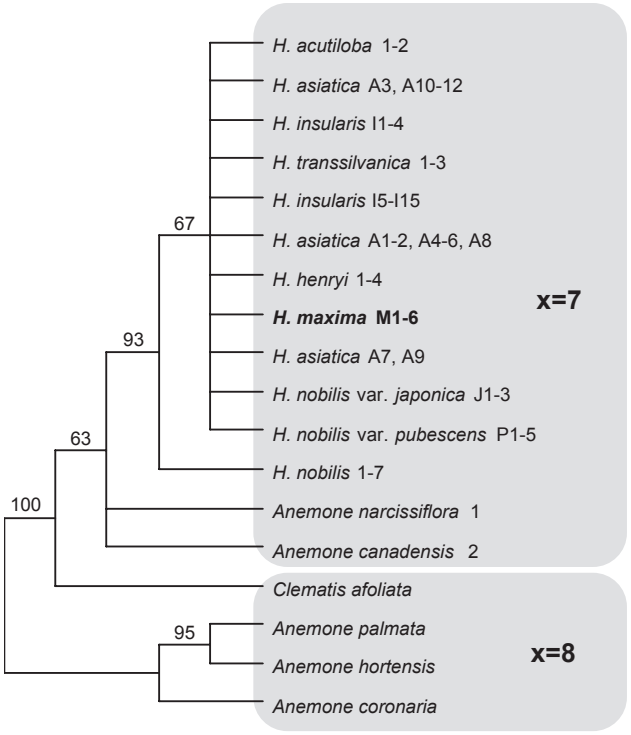

Fig. 4: Strict consensus tree based on maximum parsimony analysis of plastid *trnL-F* intron and spacer sequences. The tree is rooted with *Anemone* spp. with a basal chromosome number of x = 8. Bootstrap support values (10,000 replicates) higher than 50% are indicated above branches. The Ullung Island endemic *H. maxima* is shown in bold.

PCR products were diluted 10-fold and used for the selective amplification with different combinations of **Eco**RI and **Mse**I primers. Fluorescence-labeled **Eco**RI primers were used for selective amplification. From an initial primer trial involving 16 combinations, three selective PCR primer pairs were selected for the quality of produced bands (i.e., homogeneous band intensity, manageable number of produced bands) and used in further experiments: **Eco**-ACA/**Mse**-CAT, **Eco**-AGC/**Mse**-CTG, and **Eco**-AAG/**Mse**-CTT. Selective amplification was performed using a touchdown cycling profile starting with 20 sec at 94°C, 20 sec 65°C, 50 sec at 72°C followed by 12 cycles with a decreasing annealing temperature by 0.7°C per cycle. During the remaining 23 cycles annealing temperature was kept at 56°C. Selective amplification products were separated on 6% denaturing polyacrylamide gels with an internal size standard (Genescan 500 Rox, PE Applied Biosystems) on an ABI 377 automated sequencer. The fragment pattern in a readable range of 50-500 bp was converted into binary data represented by "0" for the absence of a band at a particular position and "1" for the presence of a band. The resulting 0/1 matrix was further analyzed using ARLEQUIN Version 1.1 (Schneider et al. 1997). Neighbor-joining analysis based on pairwise $\mathbf{F}_{ST}$ comparisons between populations was used to search for clustering of populations with NTSYS-PC Version 2.0 (Rohlf 1997).

Results

Nuclear ITS sequences

ITS alignment was 414 bp long (ITS1: 260 bp; ITS2: 154). One hundred eighty-four positions were parsimony informative among seven genera *(Anemone, Clematis, Hepatica, Knowltonia, Pulsatilla, Ranunculus,* and *Trautvetteria)*. Maximum parsimony analysis yielded eighteen equally parsimonious trees of 679 steps (consistency index CI = 0.617, retention index RI = 0.768, rescaled consistency index RC = 0.474). Six equally parsimonious trees (L = 327.224, CI = 0.784, RI = 0.867, RC = 0.680), one of which is shown in Fig. 2, were retained after successive character weighting. Ingroup relationships were similar irrespective of either choosing *Ranunculus* or *Trautvetteria* as outgroups. Monophyly of *Hepatica* is strongly supported (98% bootstrap support). Although not supported by bootstrap values, a sister group relationship of *Hepatica* and *Anemone* spp. with a basal chromosome number of x = 7 is suggested by tree topology. The genera *Clematis* and *Pulsatilla* are monophyletic (100% and 97%, respectively), whereas *Anemone* appears to be polyphyletic in the majority rule consensus trees.

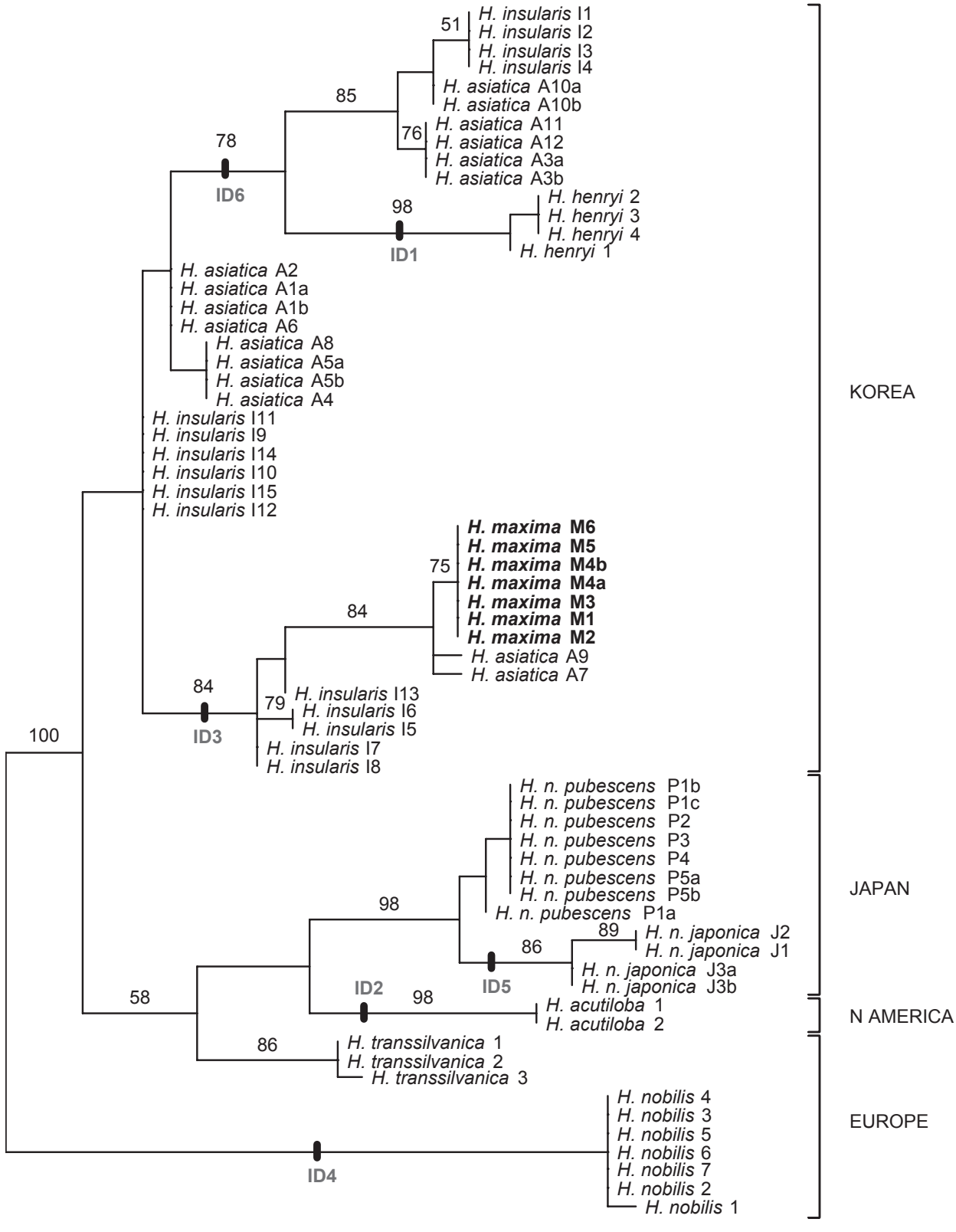

Fig. 5: Phenogram based on plastid DNA sequences (nucleotide substitutions, indels, and microsatellite data) showing the relationships among species and populations within *Hepatica*. Bootstrap support values (10,000 replicates) higher than 50% are indicated and six synapomorphic indels are mapped on the tree. Geographic origin of populations is indicated on the right margin.

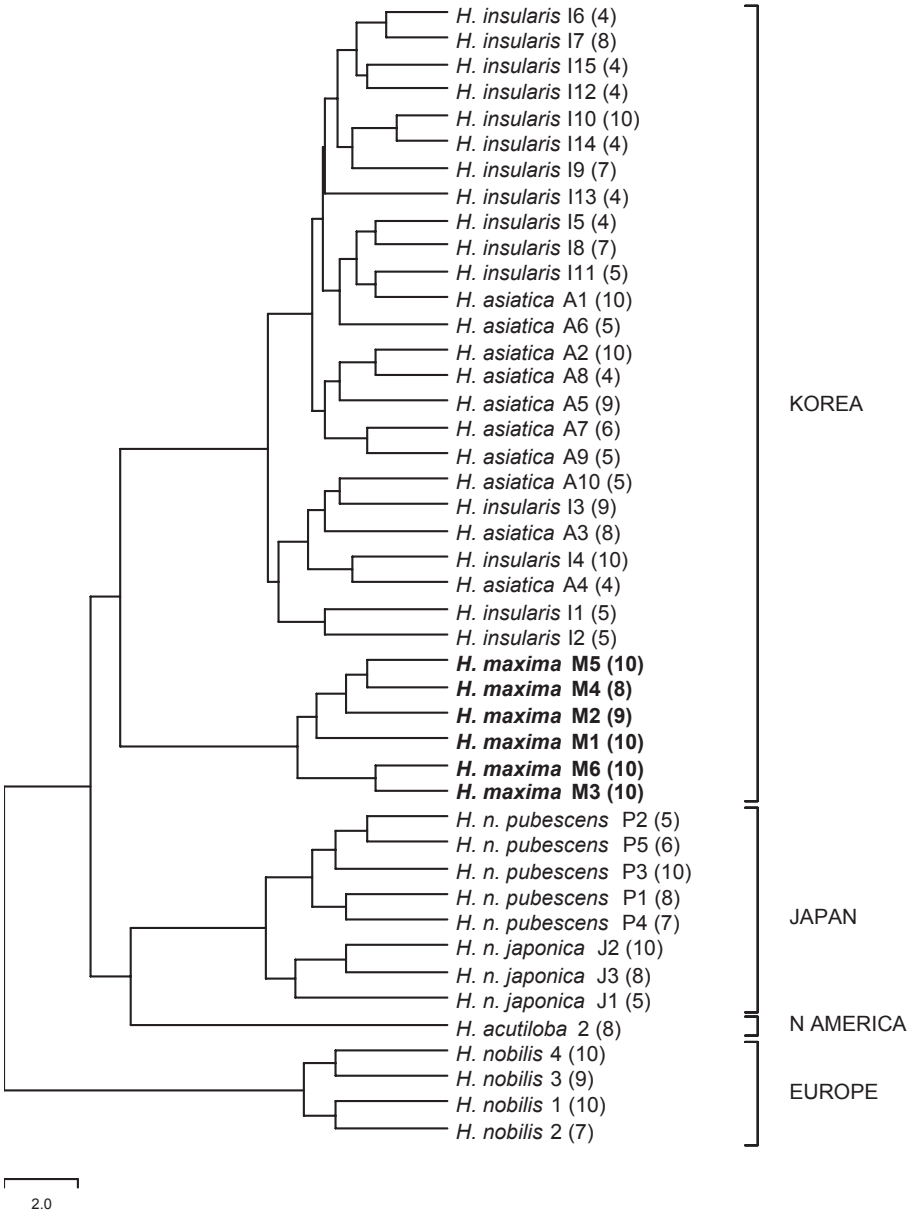

Fig. 6: Neighbor-joining analysis of populations of *H. asiatica*, *H. insularis*, *H. maxima*, *H. nobilis* var. *pubescens*, *H. nobilis* var. *japonica*, *H. acutiloba*, and *H. nobilis* based on pairwise F_{ST} values from AFLP data. Identifiers following the species names correspond to the localities of populations shown in Fig. 1B. Numbers of individuals analyzed per population are shown in parentheses.

Chloroplast atpB-rbcL spacer sequences

Alignment of the *atpB-rbcL* sequences yielded 1079 bp, 177 of which were parsimony informative. Twenty-seven equally parsimonious trees were retained with a Fitch tree length of 599 steps (CI = 0.735, RI = 0.803, RC = 0.599). Successive weighting yielded a single MP tree of a weighted tree length of 357.637 (CI = 0.912, RI = 0.939, RC = 0.856). *Hepatica* is clearly monophyletic (100% bootstrap support), but only a low level of infrageneric resolution is provided (Fig. 3). Moderate support for a clade comprising the East Asian species, *H. maxima*, *H. asiatica*, *H. insularis*, and *H. henryi*, is suggested (66%). Sister to *Hepatica* is a clade consisting of *Anemone* spp. with $x = 7$ (74% bootstrap support). Sister to this clade is *Clematis* (90% bootstrap support), whereas the remaining species of *Anemone* form a strongly supported clade together with *Pulsatilla* and *Knowltonia* (98%).

Chloroplast trnL-F intron and spacer sequences

The aligned *trnL-F* DNA matrix consisted of 1149 bp, with 115 variable and 57 parsimony-informative positions. One-hundred and sixty trees of 213 steps were retained in searches using maximum parsimony (CI = 0.934, RI = 0.880, RC = 0.822). Successive weighting reduced the number of equally parsimonious trees to 25 with a weighted tree length of 194.30 (CI = 0.976, RI = 0.955, RC = 0.932). The strict consensus tree (Fig. 4) again yields high bootstrap support for a monophyletic *Hepatica* clade (93%). All Asian and North American species and populations as well as *H. transsilvanica* from the Transcarpatians formed a clade (63%) distinct from *H. nobilis* populations from Europe. Sister to *Hepatica* are *Anemone narcissiflora* and *A. canadensis* both with basal chromosome numbers of $x = 7$. Sister to this group of $x = 7$ species is *Clematis* (100%), followed by species of *Anemone* with basal chromosome numbers of $x = 8$. Within the *trnL* intron and the *trnL-F* intergenic spacer, six synapomorphic indels (ID) of varying length (ID1 8 bp, ID2 22 bp, ID3 6 bp, ID4 4 bp, ID5 14 bp, ID6 233 bp) have been found, as well as three poly-T microsatellites (nucleotide positions 119-129, 760-771, and 1026-1034) and one poly-A microsatellite (nucleotide positions 684-692). Neighbor-joining analysis revealed additional phylogenetic resolution among species and populations within *Hepatica* (Fig. 5). According to plastid DNA data, all *H. maxima* populations appeared homogeneous and were most closely related (84% bootstrap support) to the *H. asiatica* populations A7 and A9 located in the Kangwon Province of eastern South Korea (compare with Fig. 1B). No clear structuring of *H. asiatica* and *H. insularis* populations was suggested from the plastid DNA data. Instead, populations of both species appeared extensively intermixed with each other. Both varieties from Japan, the diploid *H. nobilis* var. *japonica* and the hexaploid *H. nobilis* var. *pubescens* grouped together with high bootstrap support (98%). A possible relationship between the Japanese varieties and the North American *H. acutiloba* and the tetraploid *H. transsilvanica* from the Transcarpatians was only weakly supported (58%). The European populations of *H. nobilis* appeared to be most distantly related to all other species of *Hepatica*.

AFLP analysis

In total, 316 individual plants with 4-10 plants per population, comprising 4 populations of *H. nobilis*, 1 population of *H. acutiloba*, 3 populations of *H. nobilis* var. *japonica*, 5 populations of *H. nobilis* var. *pubescens*, 6 populations of *H. maxima*, 10 populations of *H. asiatica*, and 15 populations of *H. insularis* were analyzed with three AFLP primer combinations. Among the 297 unambiguously scoreable fragments, 239 (80.5%) were polymorphic. Relatively high levels of fixed private fragments (fragments present only in a group of individuals and missing in all other populations) were found in *H. nobilis* (7 fragments out of all polymorphic fragments; 2.93%), *H. acutiloba* (8 fragments; 3.35%), the Japanese populations (4; 1.67%), *H. maxima* (5; 2.09%), and a group consisting of the remaining Korean populations of *H. asiatica* and *H. insularis* (4; 1.67%). Neighbor-joining analysis of all populations based on a distance matrix calculated from pairwise F_{ST} values confirmed that the origin of *H. maxima* has to be found among populations located in Korea rather than in Japan (Fig. 6). Even more obvious than in the plastid microsatellite tree was the extensive intermixing of *H. asiatica* and *H. insularis* populations. All Japanese populations appeared closely related in the AFLP tree and showed at least a loose clustering with North American *H. acutiloba*.

Discussion

Phylogenetic relationships within Ranunculaceae tribe Anemoninae have been the object of phylogenetic speculation for almost a century (Ehrendorfer 1995; Starodubtsev 1991; Ulbrich 1905/6). Recently, molecular phylogenies have been produced (Ehrendorfer & Samuel 2001; Hoot 1995; Hoot et al. 1994; Schuettpelz et al. 2002) that shed new light on the relationships. Discrepancies exist regarding the classification of *Anemone*. Whereas Hoot et al. (1994) and Hoot (1995) suggested a broad concept for the genus, lumping *Hepatica*, *Knowltonia*, and *Pulsatilla* with *Anemone*, Ehrendorfer (1995) favored a narrow concept and provisionally subdivided the genus into several smaller genera. One criterion of subdividing *Anemone* is the obvious difference in basal chromosome numbers among species being either $x = 7$ or $x = 8$. In order to maintain monophyletic taxa, Hoot (1995) and Schuettpelz et al. (2002) regarded this difference as insufficient for classification purposes and suggested a broad concept for *Anemone* including both chromosome types. They based their assumptions on the resulting tree topologies obtained when *Clematis* was used as an outgroup. Ehrendorfer suggested the genus *Trautvetteria* from sister tribe Ranunculeae to be a much better outgroup candidate than *Clematis*. Although not the main focus of the current study, our results corroborate the splitting of *Anemone* based on the $x = 7/8$ divergence. When *Trautvetteria* or *Ranunculus* was used as the outroup instead of *Clematis*, a clear separation of species according to different basal chromosome numbers was obvious. This was most obvious when *atpB-rbcL* spacer sequences have been analyzed, in which *Clematis* occurred as sister to *Hepatica* and $x = 7$ *Anemone* spp. (90% bootstrap support), whereas the $x = 8$ *Anemone* spp. were basal to this clade. The karyotype of $x = 7$ in *Anemone* has been suggested to have originated from $x = 8$ by the loss of one V-type chromosome (Kurita 1958). Using *Clematis* as an outgroup would require a less parsimonious solution with the loss and subsequent acquisition of a chromosome, because the species with $x = 7$ are found in a basal (Hoot et al. 1994), and not in an advanced, position as seen also in our trees.

Our data clearly demonstrate monophyly of *Hepatica* within tribe Anemoninae of subfamily Ranunculoideae. However, it was unexpected to find only little variation in nuclear ITS and non-coding plastid DNA sequences among species and populations within the genus. Taking all sequenced regions together, only 21 (0.79%) out of 2642 nucleotide positions were variable, 18 (0.68%) of which were parsimony informative within *Hepatica*. Although Ranunculaceae are considered to be of ancient origin (Soltis et al. 1999), several members are probably the result of recent radiations, and therefore, little nucleotide variation is encountered. Recently, a molecular phylogeny of the genus *Trollius* (Ranunculaceae) was presented which shows a similar Northern Hemispheric distribution extending from Europe, the Middle East, and East Asia to North America (Despres et al. 2003). As in our study, nuclear ITS and plastid DNA sequences failed to resolve infrageneric relationships due to a low level of nucleotide variation. The authors used AFLP data, therefore,

to reconstruct better resolving phylogenies. Similar approaches have been pursued already in a variety of other plant groups (HODKINSON et al. 2000; KOOPMAN et al. 2001; ZHANG et al. 2001).

A combination of plastid DNA microsatellites and AFLP data resulted useful for reconstructing phylogenetic history in *Hepatica*. Our data suggest a basal position for the European species *H. nobilis*, and at least two additional groups of related species, one of which contains the Asian species *H. asiatica, H. henryi, H. insularis,* and *H. maxima*, and the other the Japanese varieties *H. nobilis* var. *japonica* and *H. nobilis* var. *pubescens*. The latter group also contains the North American species *H. acutiloba* and the Transcarpatian endemic *H. transsilvanica*, although bootstrap support is only moderate for this clustering. It has been questioned whether two species exist in North America (*H. acutiloba* and *H. americana*; (STEYERMARK & STEYERMARK,1960)), or if they should better be treated as two varieties of the same species. Since our results show that ITS sequences are identical for both species, they should at least be closely related. However, probably enough sequence variation exists between *H. nobilis* and the North American species to warrant specific status of their own and not to treat them as subspecies or varieties of *H. nobilis* as suggested by STEYERMARK & STEYERMARK (1960). The same holds true for the Japanese varieties, which are clearly separated genetically from *H. nobilis*, and most certainly should be recognized as a distinct species. One additional species, *H. falconeri*, an endemic in the Kashmir and Himalayan Mountains, has not been available for our study.

Several lines of evidence suggest that the Ullung Island endemic *H. maxima* has originated from founder populations located in peninsular Korea and not from Japan: (1) *atpB-rbcL* spacer sequences cluster *H. maxima* with *H. asiatica, H. insularis,* and *H. henryi* (66% bootstrap support); (2) plastid microsatellite data place all *H. maxima* populations in close vicinity with two *H. asiatica* populations from Kangwon Province of eastern South Korea (84% bootstrap support); (3) the former species share a synapomorphic indel with a few other *H. insularis* populations (84% bootstrap support), which is absent in all other populations; (4) AFLP data place *H. maxima* in a clade with *H. asiatica* and *H. insularis* whereas the Japanese populations form a separate clade. A presumptive origin for the Ullung Island endemic from peninsular Korea is not surprising because the geographical distance between Ullung Island and peninsular Korea is much shorter (137 km) than to the next closest larger land mass, the Japanese island Honshu (275 km). We have already shown for two other Ullung Island endemics, *Acer okamotoanum* and *A. takesimense,* that they have originated from progenitors from peninsular Korea and not from Japan (PFOSSER et al. 2002). However, possible colonization scenarios of plants originating from Japan cannot be excluded a priori. Examples exist whereby progenitors of Ullung Island endemics must have originated from the Japanese archipelago because close relatives are totally absent in Korea. One of the most obvious examples is in the genus *Dystaenia* (Apiaceae), which has only two species (CHOI et al. 1998), one endemic to Ullung Island (*D. takesimana*) and the other confined to Japan (*D. ibukiensis*).

Although our data clearly show that the progenitor of *H. maxima* must have been from *Hepatica* populations of peninsular Korea, the distinction between the two species, *H. asiatica* and *H. insularis*, from peninsular Korea is less obvious. From our data it is not clear if *H. insularis* is restricted to the southern coast and islands or if it also penetrates more into inland regions of Korea. A recent study on morphology and isozyme divergence in Korean *Hepatica* already indicated that *H. insularis* populations may also be found around Taegu (WOO et al. 2002). This region was also sampled in our study (population A3) and chloroplast data suggested strong ties to *H. insularis* populations from Cheju Island but also to other mainland populations. As judged from analysis of AFLP data, there is no clear pattern discriminating the two species. Alternatively, the incongruency between plastid data, which indicate several distinct plastid types, and AFLP data, suggesting no pattern among populations, may best be interpreted as a phenomenon of lineage sorting of uniparentally inherited plastids.

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