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ORIGINAL RESEARCH PAPER in RECENT DEVELOPMENTS IN TAXONOMY AND PHYLOGENY OF PLANTS

Hepatica transsilvanica Fuss (Ranunculaceae) is an Allotetraploid Relict of the Tertiary Flora in Europe – Molecular Phylogenetic Evidence

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Abstract

The *Hepatica* section *Angulosa* consists of mainly tetraploid ($2n = 28$) species that are distributed disjunctly throughout Eurasia. Karyological evidence proves the hybrid origin of the polyploid species of this section. *Hepatica transsilvanica* is a member of this species group with a conspicuous distribution restricted to the Eastern Carpathians. Based on genome size and cytotypes, the paternal parent of *H. transsilvanica* is described to be the only diploid species in section *Angulosa*, *H. falconeri*. The maternal species is hypothesized to be *H. nobilis*, a European species with entirely lobed leaves and a wider distribution area. Although the hybrid origin of *H. transsilvanica* is well documented by karyological evidence, the time of hybridization has never been studied. By using sequences of both the nuclear and plastid genome, we reconstructed the phylogenetic relationships and divergence times of *H. transsilvanica* and its parental species. The identity of the parental species is corroborated by discordant gene tree topologies of the nrITS and plastid sequences. Moreover, both gene copies of the parental species could be identified with the low-copy nuclear gene, *MLH1*. Divergence dating analysis using Bayesian phylogenetic methods strongly supported the long-term survival of *H. transsilvanica* in the Southeastern Carpathians, as the most recent common ancestor of the hybrid and parent species existed not later than the beginning of the Pleistocene, ca. 3 million years ago. These results not only highlight the biogeographic importance of the Southeastern Carpathians in the Quaternary glaciation periods, but also emphasize that Tertiary lineages could have survived in a Central European cryptic refugium.

Keywords
disjunct distribution; divergence date estimation; hybrid speciation; nrITS; *MLH1*; plastid DNA; glacial refugium; Carpathians

1. Introduction

Hepatica is traditionally known as a small genus within *Anemoninae* (Ranunculaceae) that comprises seven to nine species depending on the taxonomic concept applied (Tamura, 1993; Weiss et al., 2002). Additionally, the taxonomic rank of the genus is also unequivocal; whereas Hoot et al. (2012) subsumed *Hepatica* to the genus *Anemone*, Jiang et al. (2017) showed that *Anemone* s. l. (in this very broad taxonomic sense) is paraphyletic and separated *Hepatica* with the inclusion of the sections *Anemonidium*, *Keiskea*, and *Omalocarpus* of the genus *Anemone* s. l., which is also supported by the phylogenetic results of Liu et al. (2018). The partial inclusion of *Hepatica* and *Anemonidium* in the dated phylogeny of *Pulsatilla* (Sramkó, Laczkó, et al., 2019) revealed that their separation took place ca. 21.5 million years ago (Mya). Owing to the morphological evaluation of *Hepatica* by

Tamura (1993), together with the deep split between *Hepatica* s. s. (in a strict sense) and *Omalocarpus* + *Anemonidium* + *Keiskea* suggested by the latest phylogenetic results, we treated *Hepatica* in a strict sense (i.e., without the inclusion of related sections as listed above) at the genus level throughout this study.

Two sections are recognized within the genus *Hepatica* s. s.: the mainly diploid section *Hepatica* ($2n = 14$) is characterized by species with entirely lobed leaves, whereas the predominantly polyploid section *Angulosa* ($2n = 28$) consists of crenate-leaved species (Zonneveld, 2010). The genus shows both inter- and intracontinental disjunct distribution, with the highest species diversity in Northeast Asia (Pfosser et al., 2011). Species of the section *Angulosa* occur in Central to West China (*Hepatica henryi* Nakai and *H. yamatutai* Steward), Central Asia (*H. falconeri* Thomson), and Europe (*H. transsilvanica* Fuss). The distribution area of *H. transsilvanica* is restricted to the Southeastern Carpathians in Romania (Kliment et al., 2016), whereas *H. nobilis* Mill. – the only species of the genus in Europe that belong to the section with entirely lobed leaves – has a much wider distribution area, which covers most of Europe (Figure S1). Other species of this latter section occur in E Asia and N America (Pfosser et al., 2011, Figure 1).

Polyploid species of section *Angulosa* were proven to be allopolyploid hybrids of the only diploid but crenate-leaved Central Asian species, *H. falconeri* Thomson, and other species with entirely lobed leaves (Weiss-Schneeweiss et al., 2007; Zonneveld, 2010). The geographic distribution of these species, together with their unique cytotypes and additive genome sizes, led Weiss-Schneeweiss et al. (2007) and Zonneveld (2010) to conclude that the most probable parental species of *H. transsilvanica* are *H. falconeri* and *H. nobilis*. For its conspicuous distribution, *H. transsilvanica* was the scope of Bartha et al. (2014), who used additive polymorphic sites (APSs) (i.e., unambiguous double peaks in the direct nuclear sequences, where the single-nucleotide polymorphisms (SNPs) of both parent species occur at the same position in the hybrid specimens) of the nuclear region At103. Their results, based on APSs in this genic region, corroborated the findings of previous authors regarding the putative parental species of *H. transsilvanica*.

From a phylogeographic point of view, the divergence date of *H. transsilvanica* might have an important biogeographic implication; however, this has never been addressed. The Carpathian Mountains are important sources of genetic diversity for certain species (e.g., Mosolygó-Lukács et al., 2016; Mráz & Ronikier, 2016; Slovák et al., 2012). The long-term survival of a broad-leaved forest species in the Carpathians was tested by Bartha et al. (2015), who used *Erythronium dens-canis* L. as a model species to prove the persistence of a distinct lineage within the species in the Eastern Carpathians. Similarly, Ronikier et al. (2008) discovered a long-term isolation effect within the range of *Campanula alpina* Jacq., which shaped the genetic variation in the Eastern and Southern Carpathian populations. Moreover, such large disjunctions in species' distribution, which can be observed in *Hepatica* section *Angulosa*, could be associated with deep splits in the phylogeny (e.g., Yokoyama et al., 2000). Lendvay et al. (2016) showed that *Syringa josikae* J. Jacq., a unique species of lilac native to the Carpathians, can be validated as a Tertiary relict in the European flora. This species survived the eradication of the European Tertiary flora in the Carpathians (in the Ukrainian Carpathians and the Apuseni Mountains in Romania) (Lendvay et al., 2016) and diverged from its closest relatives ca. 1.88 Mya. The distribution patterns displayed by *S. josikae* and its closest relatives are strikingly similar to the one we can see in Asian tetraploid species of *Hepatica* section *Angulosa*.

All previous results hint at the long-term survival of *H. transsilvanica* in Europe. In our study, we investigated the origin of *H. transsilvanica* using a molecular phylogenetic approach by including the analysis of two nuclear and two plastid loci. We evaluated the phylogenetic relationship between the species and carried out a divergence date estimation to test the possible autochthon long-term survival of *H. transsilvanica* in a temporal framework. If the phylogenetic relationships reconstructed using individual gene trees mirrored an allopolyploidization event, we expect discordant topologies that could reveal the parental species of *H. transsilvanica* (Wendel & Doyle, 1998). In the case of autopolyploid origin of *H.*

transsilvanica – as alleles in the polyploid would originate from the same species – we expected gene trees to place this species concordantly in the same phylogenetic position, as sister to its closest extant relative. In both cases, the divergence age of *H. transsilvanica* could be reconstructed. A relatively old divergence of this species could possibly highlight the role of the Carpathian Mountains in the long-term preservation of this species.

2. Material and Methods

2.1. Sampling

Our field sampling focused on the distribution areas of *H. transsilvanica* and both of its most probable parental species (Bartha et al., 2014; Weiss-Schneeweiss et al., 2007), *H. nobilis* and *H. falconeri* (Table 1, Figure S1). We tried to include samples from different parts of the distribution areas of our focal species to represent the variability within species as much as we could (Figure S1). For *H. nobilis*, we included samples from the southern part of its distribution area, which was not covered with ice nor too cold for the species to survive during the Last Glacial Maximum (LGM). If this species survived glaciations in Southern European refugia (e.g., Hewitt, 1999; Taberlet et al., 1998), sampling along an eastern–western gradient should catch a significant amount of the species' variability, even with a limited sample size (Table 1, Figure S1). We analyzed one individual from each population, except for *H. falconeri*, from which species we included two samples from the same population in the analyses of nuclear DNA regions. Samples were dried in silica-gel and stored at room temperature (15–25 °C) prior to DNA extraction. To represent the Asian taxa in some of the phylogenetic analyses, the nuclear ribosomal internal transcribed spacer (nrITS) sequences of *H. asiatica* and *H. henryi* were obtained from GenBank. Newly generated sequences for the study were uploaded to GenBank (Table 1).

2.2. Choice of Genic Regions

The nrITS region is widely used in plant phylogenetics (Baldwin et al., 1995) and is described as a suitable DNA region to investigate hybridization and polyploid speciation (Wendel, 2000). When analyzing nrITS sequences, it is important to consider that different copies in the nrITS array are homogenized over time by concerted evolution (Álvarez & Wendel, 2003; Bailey et al., 2003).

As concerted evolution might distort the phylogenetic signal and we cannot assume that it is complete (Álvarez & Wendel, 2003; Feliner & Rosselló, 2007), if we want to reliably reconstruct ancient polyploidization events, nrITS should be supplemented with other – possibly low-copy – nuclear markers that are less susceptible to concerted evolution (Sang, 2002). The mutL homologue 1 gene (*MLH1*), which encodes a DNA mismatch repair protein, is documented to be single-copy in a wide range of angiosperms (Zhang et al., 2012) and was proven to be suitable to reconstruct phylogenetic relationships within the genus *Pulsatilla* (Sramkó, Laczkó, et al., 2019). Therefore, we selected this nuclear region to provide phylogenetic signal from the nuclear DNA besides the widely used nrITS.

To investigate the maternal lineages, we screened commonly used plastid genic regions for polymorphic sites. These regions were the *trn*L_{UAA} intron with *trn*L-*trn*F intergenic spacer (IGS) and four IGS regions (*trn*H-*psb*A, *pet*A-*psb*J, *psb*M-*trn*D, and *acc*D-*psa*I). After screening on a subset of samples, we selected the *trn*L-*trn*F IGS with the *trn*L_{UAA} intron and *acc*D-*psa*I for subsequent analyses. Polymerase chain reaction (PCR) primer information for the DNA regions used is given in Table S1.

2.3. Laboratory Protocols

Genomic DNA from desiccated leaves were extracted using the E.Z.N.A Plant DNA DS Mini Kit (Omega Bio-Tek; Norcross, GA, USA) following the manufacturer's instructions. PCRs were conducted in 25-µL final volume mixtures containing 1× Phusion High Fidelity Hot Start II Reaction Buffer (Thermo Scientific; Carlsbad, CA, USA), 0.2 mM of each dNTP (Thermo Scientific), 25 mM $MgCl_2$, 0.25 mg

Table 1 Geographic location of samples with GenBank accession numbers of sequences generated for the study.

Species	Sample ID	Locality	N°	E°	nrITS	*MLH*	*acc*D-*psa*I	*trn*L intron	*trn*L-*trn*F
Hepatica nobilis Schreb	*H. nobilis* 1	RO: Bihor, Nucet	46.49576	22.55516	MK550972	MT276401	MK551080	MK564172	MT276421
	H. nobilis 2	ES: Barcelona, Alpens	42.11404	2.115100	MT276393	MT276400	MT276408	MT276414	MT276420
	H. nobilis 3	IT: Genova, Vobbia	44.61509	9.014140	MT276394	MT276402	MT276409	MT276415	MT276422
	H. nobilis 4	ES: Terual, Pitarque	40.63485	−0.59698	MT276395	MT276403	MT276410	MT276416	MT276423
Hepatica transsilvanica Fuss	*H. transsilvanica* 1	RO: Ciuc, Frumoasa	46.47311	25.90105	MK550971	MK564173	MT276413	MK564171	MT276426
	H. transsilvanica 2	RO: Hunedoara, Haţeg	45.62346	22.97763	MT276399	MT276407	NA	MT276419	MT276427
	H. transsilvanica 3	RO: Braşov, Piatra Craiului	45.53632	25.21763	MT276398	MT276406	MT276412	MT276418	MT276425
Hepatica falconeri (Thomson) Steward	*H. falconeri* 1	KZ: Raiymbek, Saty	43.01455	78.25895	MT276396	MT276404	MT276411	MT276417	MT276424
	H. falconeri 2	KZ: Raiymbek, Saty	43.01433	78.25864	MT276397	MT276405	NA	NA	NA
Hepatica asiatica Nakai	*H. asiatica*				FJ597993	NA	NA	NA	NA
Hepatica henryi (Oliv.) Steward	*H. henryi*				AM267290	NA	NA	NA	NA
Anemone multifida Poir.	*A. multifida*	DE: Chemnitz (garden)			MK551023	MK564174	MK550918	MK551026	NA
Anemone sylvestris L.	*A. sylvestris*	HU: Budapest, Sváb Hill			MK551024	MK564175	MK550919	MK551027	NA

Bovine Serum Albumin (Invitrogen; Carlsbad, CA, USA), 0.2 μM of forward and reverse primers, 0.03 U of Phusion High Fidelity Hot Start II DNA Polymerase (Thermo Scientific), and 25 ng of DNA template. All PCR thermal cycling regimes were performed as specified in Laczkó et al. (2019), except for that of the *MLH1* gene, for which we used the same PCR profile as for the nrITS, but set the annealing temperature to 57 °C. Successfully amplified PCR products were sequenced using a commercial service (Macrogen Inc., South Korea).

2.4. Reconstruction of Phylogeny and Divergence Date Estimation

As our target species is a known hybrid, we checked all raw electropherograms by eye, not just for obvious sequencing errors, but also for APSs. We trimmed off both ends at the same position and aligned the sequences using MUSCLE v.3.8.31. (Edgar, 2004), then phased ambiguous sites of the nuclear sequences using PHASE v.2.1.1. (Stephens et al., 2001; Stephens & Scheet, 2005) with default options, for which the format conversion was performed using the SeqPHASE online tool (Flot, 2010). The phased sequences of such samples were included in the subsequent analyses as sequence version "a" and "b" of the same samples. The PHI statistics (pairwise homoplasy index) (Bruen et al., 2006) to test for possible recombination events in the nrITS and *MLH1* sequences was conducted in SplitsTree v.4.14.4. (Huson & Bryant, 2006).

As the molecular evolution of different genic regions can be totally different, in the case of hybrid speciation, concatenating them would easily lead to ambiguous phylogenetic tree topologies. To avoid such a situation, we preferred to study the phylogenetic pattern of gene trees for our selected genic regions separately, and interpreted the conflict between the gene trees as possible evidence for hybrid speciation (Wendel & Doyle, 1998). The exception was the plastid sequences (i.e., *trn*L-*trn*F and *acc*D-*psa*I), which are tightly linked and evolve as a single unit representing the maternal lineage (Mogensen, 1996); therefore, we concatenated them into a single "plastid" dataset.

Phylogenetic relationships between our focal species were reconstructed by both maximum likelihood (ML) and Bayesian methods. For the ML analysis, we used the online version of PhyML 3.0 (Guindon et al., 2010) with smart model selection (SMS) (Lefort et al., 2017). To assess branch support, besides the nonparametric Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT) and bootstrap (bs), parametric aBayes values were calculated; aBayes exhibits the highest statistical power, whereas nonparametric tests are more conservative but (especially aLRT) may be more robust if not all assumptions are met (Anisimova et al., 2011). Nonparametric bootstrap support values were calculated after 1,000 iterations. We interpreted only those branches that were supported by at least two statistics and considered a branch supported if SH-aLRT or aBayes >0.95, and bs >70%.

We estimated the species' divergence times using BEAST v.2.6.1. (Bouckaert et al., 2019) for nrITS, *MLH1*, and the plastid dataset separately. To test the presence of a strict molecular clock, we compared the marginal likelihoods of runs that used the strict clock with those that used the uncorrelated relaxed clock. We assessed marginal likelihoods using nested sampling (NS) to compare the probabilities of the clock models by Bayes factors (BFs) – a method demonstrated to have a high statistical power (Maturana-Russel et al., 2018). NS used 10 particles, a chain length of 100 million, and a subchain length of 10 million. To find the optimal substitution model, we applied the Bayesian model test (Bouckaert & Drummond, 2017) simultaneously in each run.

For secondary dating, we placed a calibration point at the root, taking the estimates of Wang et al. (2016) as a normal prior with a mean of 27.6 Mya and a sigma of 1.4. Weiss-Schneeweiss et al. (2007) showed that the speciation events within *Hepatica* occurred in the Pliocene, ca. 3 Mya, but their phylogeny was not well resolved. Divergence dating used a general clock rate and – as admitted by the authors (Weiss-Schneeweiss et al., 2007) – an external calibration point that should be used with caution (see below). Therefore, we used their estimations with a higher margin of error than described and placed a normal prior on the age of *Hepatica* with a

mean of 3.0 and a sigma of 4.0, resulting in a prior age ranging from the present to 10.4 Mya. The gene trees reconstructed by ML were specified as starting trees, but without any multimonophyletic constraints. BEAST v.2.6.1. (Bouckaert et al., 2019) was run two times on each dataset for 500 million generations, saving every fifty thousandth sample. Plastid regions were allowed to have a different substitution model, but the clock rate and the resulting trees were linked in all analyses. We checked the runs for effective sample size (ESS) and convergence using Tracer v.1.6.0., which were accepted if the ESS was greater than 200 with 10% burn-in. We combined runs with LogCombiner v.2.6.1. (Bouckaert et al., 2019) and checked postburn-in trees with Densitree v.2.2.7. (Bouckaert et al., 2019). We reconstructed the maximum clade credibility (MCC) tree with TreeAnnotator v.2.6.0. (Bouckaert et al., 2019). We interpreted not just the MCC tree, but the 95% highest posterior density (HPD) topologies that were estimated by TreeLogAnalyser v.1.10.4. (Suchard et al., 2018).

3. Results

3.1. Sequence Variation

The aligned nrITS data matrix consisted of 568 positions, 90 of which were variable and 58 informative, including the outgroup. Within the ingroup, we identified 19 variable and 10 informative sites. We found only one double peak in the ITS1 region of "*H. transsilvanica* 2" (Table S2), which was not an APS, but was successfully phased. The PHI statistics found no evidence for recombination ($p = 0.888$).

The *MLH1* matrix was 318-bp long. Forty-seven sites were variable and 24 informative across the entire data set; within the ingroup, we found nine variable and eight informative SNPs. We found four positions where double peaks could be identified (Table S2), but these were restricted to the sequences of *H. transsilvanica*. All ambiguous sites were successfully phased, except for one (position 41 in the aligned matrix) that was only found in "*H. transsilvanica* 3" and coded by the IUPAC nucleotide ambiguity codes in subsequent analyses. We found two positions that showed an additive pattern between *H. nobilis* and *H. falconeri*. One of them was a consistent APS within all *H. transsilvanica* samples, whereas the other appeared to be additive only in "*H. transsilvanica* 2" and "*H. transsilvanica* 3." The PHI statistics showed no evidence for recombination ($p = 0.055$).

Aligned length of the concatenated *acc*D-*psa*I, the *trn*L_{UAA} intron and the *trn*L-*trn*F IGS (i.e., the plastid dataset) was 1,275-bp long and contained 33 variable and 30 informative SNPs in total. Within the ingroup, we found eight variable positions, four of which appeared to be informative.

3.2. ML Phylogenetic Reconstruction

All of the gene trees reconstructed by PhyML separated the outgroup species with high certainty but resulted in different topologies in the placement of *H. transsilvanica* (Figure 1).

SMS identified the GTR model as the best fitting model of sequence evolution for nrITS. This analysis identified *H. asiatica* and *H. henryi* as sister taxa with high support. Samples of *H. nobilis* were placed on a short branch and formed a metaphyletic group, where the separation was supported only by SH-aLRT. The separation of *H. falconeri* and *H. transsilvanica* as sister species received high statistical support (Figure 1A).

For the *MLH1* dataset, the HKY85 + G model was identified as the best fitting by SMS. Two sequences of *H. transsilvanica* ("*H. transsilvanica* 2b" and "3b") were grouped together with *H. falconeri* (Figure 1B). As we move toward the tip of the tree, an intermediate group ("*H. transsilvanica* 1b" and "*H. transsilvanica* 1a–3a") within *H. transsilvanica* could be identified, although their separation was only supported by SH-aLRT and aBayes (SH-aLRT: 1, aBayes: 0.97). The separation of *H. nobilis* on a relatively longer branch received high statistical support. In sum, *MLH1* clearly placed all phased *H. transsilvanica* samples as intermediates between its putative parents, although the phylogenetic relationship between these intermediate lineages could not be resolved with statistical confidence (Figure 1B).

The best fitting nucleotide substitution model for the plastid dataset was GTR + G. All branches leading to species received high statistical support. Although *H. transsilvanica* and *H. nobilis* are placed as sister taxa, the monophyly of this clade could not be confirmed by statistical tests, except for by SH-aLRT (Figure 1C).

3.3. Estimation of Divergence Dates

We successfully estimated the divergence time of the target species using a Bayesian approach as implemented in BEAST v.2.6.1. (Bouckaert et al., 2019). The advantage of this approach is that it is not only able to return the MCC tree, but the 95% HPD of tree topologies can also be examined.

For the nrITS dataset, NS supported the presence of the strict clock over the uncorrelated relaxed clock model, whereas for the *MLH1* sequences, the presence of a strict molecular clock was rejected. Standard deviations of the marginal likelihood estimates for the nrITS and *MLH1* sequences were much lower than the differences in marginal likelihood values (Table S3). NS was not able to distinguish between the likelihood of clock models for the plastid dataset, as the standard deviation of likelihood values were approximately half that of the BF comparing the models (Table S3). Given that the 95% HPD of trees and node ages were overlapping for the plastid dataset with different molecular clock settings, we present our results based on the uncorrelated relaxed clock, which had a slightly better marginal likelihood in our NS analysis.

The 95% HPD tree set of the nrITS dataset consisted of 24 unique trees, all of which had the same backbone topology (Figure 2A). The estimated root age was 29.4 million years (My) (95%HPD = 27.5–31.4 Mya). The first branch within *Hepatica* separated *H. asiatica* and *H. henryi* as sister species, which shared a most recent common ancestor (MRCA) with *H. nobilis*, estimated to lived ca. 6.7 Mya (95%HPD = 4.3–9.7 Mya) in the Messinian age of the Miocene epoch. The next diversification event [5.2 Mya (95%HPD = 4.3–9.7 Mya) in the early Pliocene] separated *H. nobilis*. This analysis revealed a deep split within *H. nobilis*, as the MRCA for the samples of this species could be found roughly at the transition of the Zanclean and Piacenzian ages of the Pliocene, 3.8 Mya (95%HPD = 1.4–6.2 Mya). *Hepatica transsilvanica* was placed as sister species to *H. falconeri*. The split between these two species is estimated to be 3.2 My old (95%HPD = 3.0–7.8 Mya), which corresponds to the late Pliocene. The 95% HPD trees differed from each other only in their structure within *H. transsilvanica*, where no equivocal structure could be identified.

The same analysis of the *MLH1* sequences contained 15 unique trees and revealed two different backbone topologies (Figure 2B). With an estimated root age of 27.8 My (95%HPD = 25.9–29.8 Mya), the divergence age of *Hepatica* appeared to be 6.4 My old (95%HPD = 3.1–10.0 Mya). In agreement with the MCC tree, 78% of the trees placed three copies (copies "a") (Figure 2B) of three different samples of *H. transsilvanica* as sister to *H. nobilis* with an MRCA age of 4.7 My (95%HPD = 2.1–8.6 Mya). The remaining trees identified *H. nobilis* as sister to all the other samples and copies "a" as sister to all other sequences of *H. falconeri* / *H. transsilvanica* with an MRCA age of 5.7 My (95%HPD = 2.4–8.6 Mya). The other three copies of *H. transsilvanica* (copies "b") (Figure 2B) are grouped together with *H. falconeri*, although only two of them ("*H. transsilvanica* 2b" and "*H. transsilvanica* 3b" from Haţeg and Piatra Craiului, respectively) formed a monophyletic clade. The divergence of "*H. transsilvanica* 1b" from "*H. transsilvanica* 2b" / "*H. transsilvanica* 3b" was estimated to be 3.5 My old (95%HPD = 0.8–6.8 Mya), whereas the MRCA of *H. falconeri* and "*H. transsilvanica* 2b" / "*H. transsilvanica* 3b" can be found at the transition of the Pliocene/Pleistocene 2.4 Mya (95%HPD = 0.2–4.8 Mya).

We found two alternative topologies in the 95% HPD tree set of plastid sequences, which consisted of 24 trees (Figure 2C). The root age of the tree was 26.5 My (95%HPD = 24.5–28.4 Mya). The MCC tree and 63% of the tree set placed *H. falconeri* as sister to *H. nobilis* / *H. transsilvanica*, with an MRCA in the middle Pliocene, 3.5 Mya (95%HPD = 1.8–5.4 Mya). Considering this topology, *H. transsilvanica* diverged from *H. nobilis* 2.4 Mya (95%HPD = 1.2–3.7 Mya), roughly

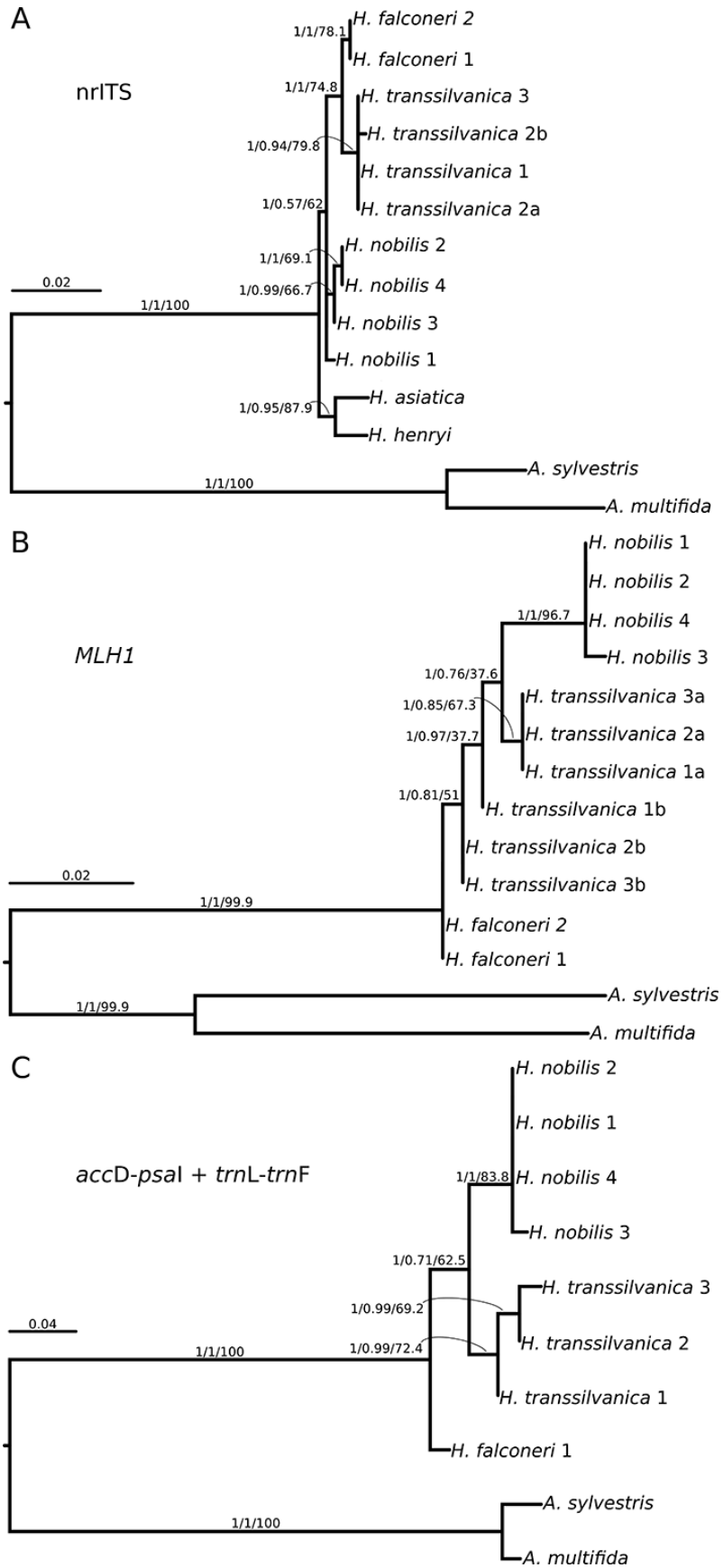

Figure 1 Phylogenetic relationships reconstructed by PhyML using the smart model selection (SMS) for (**A**) nrITS, (**B**) *MLH1*, and (**C**) concatenated plastid sequences. Statistical support values above branches correspond to Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT)/aBayes/bootstrap (bs) values.

at the transition of the Pliocene/Pleistocene, whereas the alternative topology groups *H. falconeri* and *H. transsilvanica* together on a short branch with an MRCA of 3.2 My (95%HPD = 1.5–4.9 Mya).

4. Discussion

In this study, we investigated the phylogenetic position and divergence time of *H. transsilvanica* and its presumed parental species. Our results based on a wider set of genic regions agree with Weiss-Schneeweiss et al. (2007) in the placement of our focal species. Based on nrITS, species occurring in Central and Eastern China (*H. asiatica* and *H. henryi*) are placed on a distinct lineage sister to the rest. European taxa (*H. nobilis*, *H. transsilvanica*) are assessed to be monophyletic with the Central Asian *H. falconeri*. Phylogenetic analysis of nrITS placed *H. nobilis* as sister to *H. falconeri* / *H. transsilvanica*. In contrary, plastid data suggest that *H. transsilvanica* is closest to *H. nobilis* and *H. falconeri* is the sister taxa of European species. Although Zonneveld (2010), based on its smallest genome size, hypothesized *H. falconeri* to be the most probable basal taxon within *Hepatica*, this assumption is not supported by the placement of *H. falconeri* on our nrITS tree (Figure 2A), as Eastern Asian species clearly diverged earlier.

The divergence times estimates found in our analyses apparently support the previous finding of Weiss-Schneeweiss et al. (2007) that the Pliocene played an important role in the speciation events of *Hepatica*, but we found older estimated dates. Previously described crown age (i.e., the MRCA age of a given group of samples) of *Hepatica* based on nrITS sequences and a wider taxonomic sampling lies between ca. (4.3–)2.5(−0.8) and (0.9–)0.5(−0.2) Mya, but our estimation suggests a 6.7-My-old (95%HPD = 4.23–9.7 Mya) divergence for the ingroup (i.e., *Hepatica* s. s.), even though we used a narrower set of samples. A similar estimate is achieved using *MLH1* sequences but without the inclusion of any East Asian species. The discordance in the timing of isolation events between our study and the previously published analysis might be caused not only by the different genic regions used – as Weiss-Schneeweiss et al. (2007) also used nrITS – but the different strategy and calibration point for divergence dating. Weiss-Schneeweiss et al. (2007) used a general clock rate for the genic regions included in their study and the age of Ullung Island – the distribution range of *H. maxima* Nakai – as an external calibration point (1.8 Mya), which can be interpreted as both the minimum or maximum age. Moreover, instead of nonparametric rate smoothing (NPRS) and penalized likelihood (PL), we estimated species divergence times using a Bayesian phylogenetic method, as implemented in BEAST v.2.6.1., and applied a secondary calibration point suggested by the fossil calibrated phylogeny of Wang et al. (2016). The estimated divergence times in our study are in accordance with the divergence time of other Eurasian species with similar distribution patterns. The fossil evidence clearly demonstrates that such species were distributed much more widely throughout Eurasia before the Quaternary (e.g., Lendvay et al., 2016; Yokoyama et al., 2000).

These results suggest that a re-evaluation of the evolutionary history of the genus and the possible driving forces of speciation, including hybridization within *Hepatica*, should be the subject of future studies. Another intriguing result might be the crown age for *H. nobilis* found in our analyses (mean = 3.8 Mya, 95%HPD = 1.4–6.2 Mya) that correspond to the middle Pliocene. A phylogeographic study using *H. nobilis* as a model species could potentially reveal important aspects of the phylogeography of the European temperate flora; this broad-leaved forest species is present in Europe from the onset of the Quaternary period. These evolutionary relationships, both at the genus and species levels, could be potentially investigated by the usage of a genomic approach that can provide an in-depth phylogenetic resolution, even if genetic differentiation is shallow (e.g., Sramkó, Paun, et al., 2019; Twyford & Ennos, 2012).

Still, our results provide evidence of the origin of *H. transsilvanica*. The hybrid origin of this species is corroborated by the incongruent phylogenetic placement (Wendel & Doyle, 1998) of our samples based on nrITS and plastid sequences, as already postulated by the molecular results of Weiss-Schneeweiss et al. (2007).

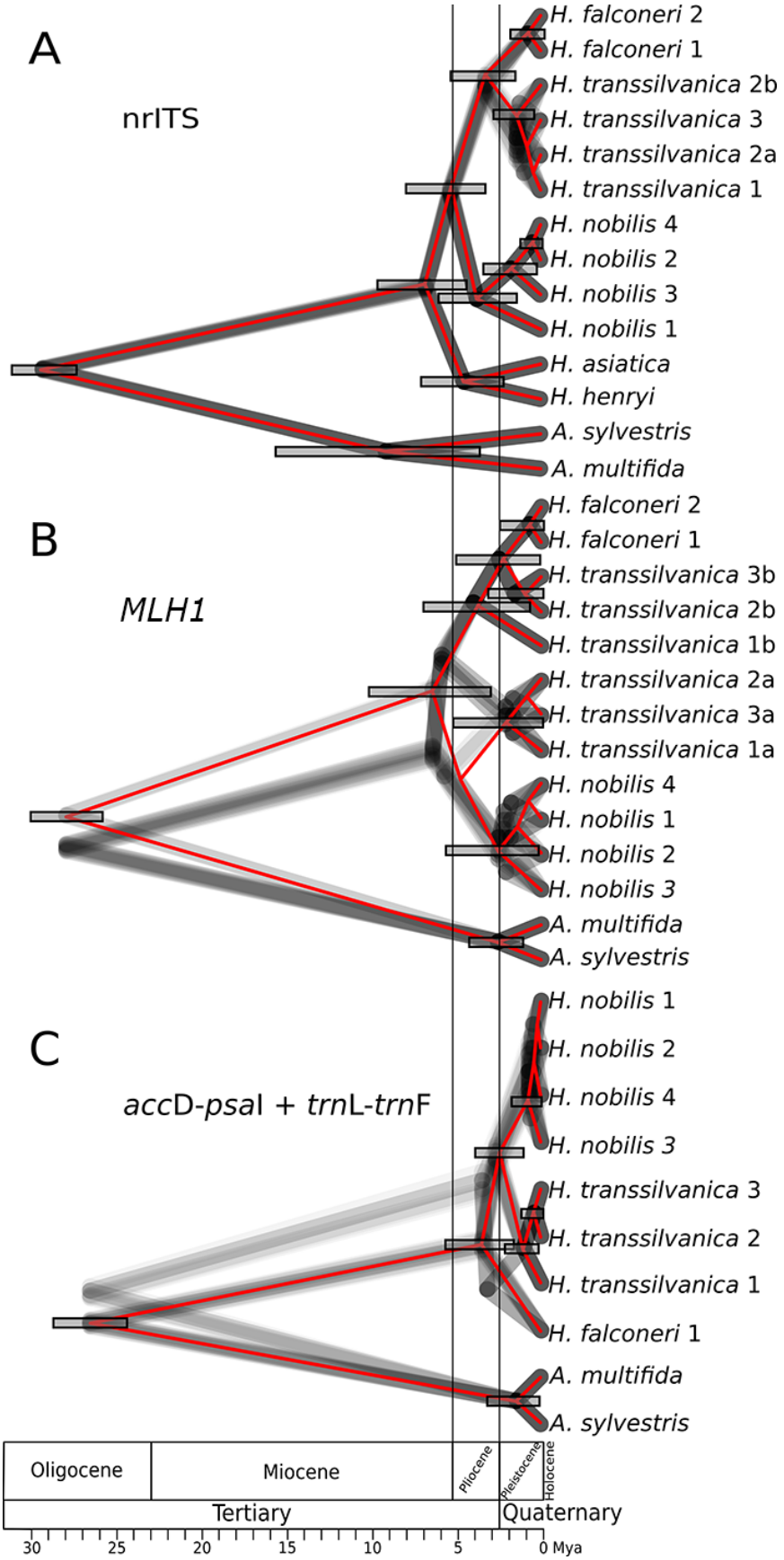

Figure 2 Dated phylogeny of a setof species as reconstructed by BEAST v.2.6.1. 95% highest posterior density (HPD) topology is visualized as grey overlapping phylograms. The maximum clade credibility (MCC) tree is projected on the tree set in red color. Error bars are only given for nodes with a minimum posterior of 0.5 and represent the 95%HPD age of the node. The geographic time scale is placed below the tree in the corresponding places.

Using the low-copy nuclear region *MLH1*, the hybrid speciation event could be reconstructed directly, as the MCC tree placed copies "a" and "b" of *H. transsilvanica* on two lineages, each affiliated to the lineage of the putative parental species (Figure 2B), *H. nobilis* and *H. falconeri*. The paraphyletic placement of copies "b" and the topological incongruence of copies "a" (Figure 2B) might be the result of reticulation, although the PHI statistics did not find significant evidence of recombination ($p = 0.055$). The statistical uncertainty of placement of copies "a" can be the result of molecular evolutionary processes (e.g., gene conversion) shifting maternal gene copies toward the paternal copies. The overall paraphyly of *MLH1* copies (Figure 2B) might be a faithful representation of a 3-My-old gene conversion process in an allopolyploid species. Our analysis of plastid sequences (Figure 2C) supported the hypothesis (Weiss-Schneeweiss et al., 2007) that *H. nobilis* is the maternal parent of *H. transsilvanica*. It seems unlikely that another Asian taxon might have played a role in the speciation of *H. transsilvanica* as the divergence of the hybrid species is estimated to be much younger than the MRCA of *H. asiatica* and *H. nobilis*, two species with entirely lobed leaves.

The long-term survival of *H. transsilvanica* in the Southeastern Carpathians is strongly supported by our results. Dating analyses of three genetic regions independently estimated that the isolation of *H. transsilvanica* from its paternal parent (i.e., *H. falconeri*) took place 3.5–3.2 Mya, as assessed from *MLH1* "b" copies and nrITS, respectively. The maternally inherited plastid regions revealed the separation of *H. transsilvanica* from its maternal parent (that is, *H. nobilis*) took place 2.4 Mya. The significantly lower effective population size of the plastid genome compared to the tetraploid nuclear genome (Schaal et al., 2000), together with the lack of recombination, allow plastid genes to respond more instantly to isolation and give a more accurate date of divergence. Thus, the isolation of the hybrid *H. transsilvanica* coincides with the start of Quaternary glaciation cycles. Conclusively, all of our analyses using the molecular clock suggested that the MRCA of *H. transsilvanica* and the parental species existed no later than the early Pleistocene, a period during which a large portion of the Tertiary flora in Europe was reported to have been eradicated (Svenning, 2003; Willis & Niklas, 2004).

On this background, it is safe to conclude that *H. transsilvanica* is a relict of the Tertiary Flora that was able to survive the Quaternary climatic changes in the Southeastern Carpathians. The biogeographic importance of this region has been proven before (Mráz & Ronikier, 2016); however, the temporal aspect of phylogeography was not always reconstructed (e.g., Bartha et al., 2015; Ronikier et al., 2008). Similarly to the example of *Syringa josikae* (Lendvay et al., 2016), our results not only highlight the biogeographic role of the Carpathians during the Quaternary glaciations but show the potential of this mountain range in the long-term preservation of Tertiary lineages in Central Europe. Although exact phylogeographic relationships within *H. transsilvanica* remain to be revealed, its refugium could be associated with calcareous bedrocks. The long-term climatic stability of this mountain range made the survival of endemic species possible in heterogeneous habitat types (see Hurdu et al., 2016 for details). Several broad-leaved forest "dacian" endemic species (i.e., those *Fagion* species with a distribution centered on Transylvania), such as *Aconitum moldavicum* Hacq. ex Rchb., *Dentaria glandulosa* Waldst. & Kit., *Symphytum cordatum* Willd., and *Pulmonaria rubra* Schott, etc., can similarly be remnants of a stable glacial refugium in the Eastern Carpathians region (Hendrych, 1981).

5. Supporting Material

The following supporting material is available for this article:

- Figure S1. Geographic location of samples collected for this study.
- Table S1. Primer names and sequences used to amplify and sequence DNA regions.
- Table S2. Sites with double peaks in the nuclear sequences.
- Table S3. Marginal likelihood values estimated by nested sampling for clock models.

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