

The Colchic region as refuge for relict tree lineages: cryptic speciation in field maples

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Abstract: ITS sequences from isolated populations of *Acer* in the Colchic region allow direct assumptions about the origin, evolution, and radiation of one of the most abundant West Eurasian tree species, *Acer campestre* L., the field maple. We inferred the molecular evolution of the internal transcribed spacers ITS1 and ITS2 of the nuclear-encoded 35S rDNA cistron in *Acer campestre* using a range of methods including sequence- and distance-based phylogenetic networks and motif analyses. The data basis for our inference are 344 sequenced clones from 51 individuals of *A. campestre*, essentially covering the modern distribution range of the species, and 27 individuals representing other species of *Acer* section *Platanioidea*. Our results indicate that *Acer campestre* comprises 2 cryptic species, 1 of which is confined to North-East Turkey and South-West Georgia (ancient Colchis region). Detailed analysis shows that the Colchic genotype is generally more similar to other species of the section than the main genotype of *Acer campestre* sampled from North Turkey to the Pyrenees. In the light of the fossil record and general differentiation patterns in West Eurasian *Acer*, we conclude that the Colchic genotype represents a survivor of the initial radiation within section *Platanioidea*, predating the formation of modern species.

Key words: *Acer campestre*, *Acer orthocampestre* sp. nov., biogeography, Colchis, cryptic speciation, East Asia, genotaxonomy, ITS region, phylogenetic networks, West Eurasia

1. Introduction

The genus *Acer* is the second-largest tree genus in the northern hemisphere in terms of the number of accepted species (van Gelderen et al., 1994; de Jong, 2002). Intrageneric classifications of the genus based on morphology and chemotaxonomy have been unstable (Pax, 1885, 1886; Pojárkova, 1933; Ogata, 1967; de Jong, 1976; Delendick, 1981). More recently, phylogenetic studies based on broadly sampled molecular data (Grimm et al., 2006; Renner et al., 2008) recognised clearly circumscribed groups, which are in good agreement with the latest morphological revision of the genus by de Jong (1994, 2002; see Grimm, 2003, Table 4-1 for an overview). The basic chromosome number in *Acer* is $x = 13$. Species of section *Platanioidea* as most other species in *Acer* have the diploid chromosome number $2n = 26$. Tetraploids and hexaploids occur in a few species (van Gelderen et al., 1994). The 2 most important sections, regarding abundance and molecular and morphological diversity, are sections *Acer* (see Grimm et al., 2007) and *Platanioidea* according to de Jong's classification. Section *Platanioidea* comprises 3 to 5 species in West Eurasia (*A. campestre* L., *A. cappadocicum* Gled., *A. divergens* K.Koch & Pax ex Pax [variously treated as subspecies of *A. cappadocicum*], *A. lobelii* Ten. [ditto],

A. platanoides L.) and up to 12 species in East Asia (Xu et al., 2008; Flora of China, 2014). In West Eurasia, species of section *Platanioidea* are widespread. *Acer campestre* L. is a very common accessory element in well-drained forests and riparian stands with a range extending from West Asia via Europe into North Africa. The species is typically found along forest-edges in fully humid, warm-temperate climates (*Cfa*, *Cfb* climates according to the Köppen climate classification; Kotttek et al., 2006) and widely cultivated. The more cold-tolerant *A. platanoides* L. extends northwards to Tromsø, Norway, and in Finland to 62°N latitude (border of *Dfb* to *Dfc* climate; van Gelderen et al., 1994; Anderberg and Anderberg, 2010). Species of the *A. cappadocicum* complex, including 3 (sub)species in West Eurasia and 1 in East Asia, have a disjunct distribution. *Acer lobelii* is restricted to South Italy (*Cfa* climate), *A. cappadocicum* s.str. is widespread in the Euxinian-Hyrcanian forest region (*Csa* and *Cfa* climates), and *A. divergens* has a highly restricted distribution in North-East Turkey (Browicz and Zieliński, 1982). The East Asian (sub)species, *A. cappadocicum* and *A. cappadocicum* subsp. *sinicum* (Rehd.) Hand.-Mazz., are found on the Himalayan foot hills and adjacent mountain ranges of South-West China at 1500–3000 m a.s.l. (Flora of China, 2014; mostly *Cwa*, *Cwb* climates).

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The species of the section are leaf-morphologically diverse, which is reflected by the number of synonyms and infraspecific taxa. While pentalobate or septalobate leaves are commonly found in species of section *Platanoidea*, trilobate leaves occur in *A. divergens* and have been reported for *A. campestre* [*A. campestre* subsp. *marsicum* (Guss.) Hayek]. Nearly unlobed leaves occur in some subspecies of the *A. longipes* Rhed.-*A. amplum* Rehd. complex (*A. amplum* subsp. *amplum*, *A. catalpifolium* Rhed., *A. firmianoides* Cheng), putative close relatives of *A. cappadocicum*. Common characteristics of section *Platanoidea* are the flattened samaras with wide-spread wings and the occurrence of laticiferous tissues (van Gelderen et al., 1994). Unique morphological features of *A. campestre* within section *Platanoidea* are the occurrence of persistent-tomentose leaves in addition to glabrous ones and corky wings on (young) branches. Genetically, *A. campestre* is unique by showing a conspicuously high intra- and interindividual variability in the internal transcribed spacers ITS1 and ITS2 of the 35S ribosomal DNA repeats, not linked to pseudogeny (Grimm et al., 2006). Nevertheless, all ITS variants of *A. campestre* exhibited unique sequence motives in generally length-polymorphic and divergent regions, which are highly diagnostic in the genus from species to section levels (Grimm, 2003; Grimm et al., 2007).

In 2006, we collected individuals of *A. campestre*, *A. cappadocicum*, and *A. divergens* growing in close vicinity in the valley of a tributary of the Çoruh River, North-East Turkey. The ITS sequences of these individuals showed a new unique sequence type that lacks some of the diagnostic sequence characteristics of *A. campestre* from elsewhere in West Eurasia but occur in individuals from West Georgia. In the present paper, we characterise the new sequence types in the light of general intra- and interspecific ITS divergence in section *Platanoidea*. Cryptic speciation in *Acer* and the evolutionary significance of the newly discovered genotype are discussed. We propose a new species, *Acer orthocampestre*, to accommodate the unique genotype of the Colchic region.

2. Materials and methods

2.1. Sampling

Material of *A. campestre* and other species of section *Platanoidea* in West Eurasia was collected during several field trips by the authors and colleagues between 1997 and 2007 (Table). Vouchers of sequenced individuals are kept in S and ISTO. The material can be provided upon request by the second author.

Table. Sampled specimens of *Acer* section *Platanoidea*.

Species	Voucher	Clone no.	Locality
<i>A. campestre</i>	Schlee MS01132-1...3 (TUB)S	ca03	Italy, border of Liguria to Toscana; Passo della Cisa
<i>A. campestre</i>	Schlee MS01139 (TUB)S	ca14	France, Alpes-de-Haute-Provence; S of river Petit Buech, Veynes, W of Gap
<i>A. campestre</i>	Denk TD01142 (TUB)S	ca15	Austria; Bisamberg, western slopes, Vienna
<i>A. campestre</i>	Denk TD01143 (TUB)S	ca16	Bulgaria; surrounding of Šumen
<i>A. campestre</i>	Schlee MS01164...166 S	ca17	France, Montagne de la Baume; S-exponated gorge at the NW slope, 1 km of La Baume, E of Sisteron; 3 individuals
<i>A. campestre</i>	Grimm GG01189 (TUB)S	ca18	Germany, Baden-Württemberg; Stauden, Tübingen
<i>A. campestre</i>	Grimm & Blessing GG01192 (TUB)S	ca19	France, dépt. Aude; Vallée du Célé, next to riverbed, 2.8 km after Quillan along route D117
<i>A. campestre</i>	Grimm & Blessing GG01246 (TUB)S	ca21	France; P.R. Volcans d'Auvergne; 1 km of St. Anastaise (route to Valbeleix)
<i>A. campestre</i>	Schlee MS01247...249 (TUB)S	ca22	Germany, Rheinland-Pfalz; Burg Eltz
<i>A. campestre</i>	Grimm & Blessing GG01250 (TUB)S	ca23	Luxemburg; Mühltal
<i>A. campestre</i>	Grimm & Blessing GG01251/...52 (TUB)S	ca24	France; Vallée de la Cisse; 2 individuals
<i>A. campestre</i>	Grimm & Blessing GG01254 (TUB)S	ca26	France, Auvergne; nearby St. Nectaire
<i>A. campestre</i>	Grimm & Blessing GG01255/...56 (TUB)S	ca27	France, dépt. Lot; upper Vallée du Célé, near route D41

Table. (Continued).

Species	Voucher	Clone no.	Locality
<i>A. campestre</i>	Grimm & Blessing GG01257...59 (TUB)S	ca28	France, Cevennes; Gorge de la Jonte, 2 km from Meyruais along route D996
<i>A. campestre</i>	Grimm & Blessing GG01260 (TUB)S	ca29	France, Cevennes; Causse Noir, SW of Meyruais, directly beneath the plateau
<i>A. campestre</i>	Grimm & Blessing GG01261...63 (TUB)S	ca30	France, dépt. Gard; Vallée de l'Hérault, near Eglise St. Cros, between St. Hippolyte du Fort (4 km) and Cros; 3 individuals
<i>A. campestre</i>	Grimm & Blessing GG01264 (TUB)S	ca31	Spain, Catalonia; Val del Llobregat (Spanish Pyrenees) ~3 km E of Guardida de Bergueda
<i>A. campestre</i>	Grimm & Blessing GG01366 (TUB)S	ca33	France; Vallée de l'Herault; along D169, Cros to St. Hippolyte, near river bed
<i>A. campestre</i>	Grimm & Blessing GG01367 (TUB)S	ca34	France; Vallée de l'Herault; along D169, Cros to St. Hippolyte, near river bed
<i>A. campestre</i>	Grimm & Blessing GG01369 (TUB)S	ca36	France, dépt. Cantal; near Vic-sur-Cère, near route from St. Jacques to Thiezac
<i>A. campestre</i>	Grimm & Blessing GG01370 (TUB)S	ca37	France, dépt. Cantal; near Vic-sur-Cère, near route from St. Jacques to Thiezac
<i>A. campestre</i>	Schlee MS01374 S	ca38	Italy, Piemonte; Parco Nazionale della Collina di Superga
<i>A. campestre</i>	Denk & Grimm 2006089 S	ca39	Turkey; Bolu to Abant Gölu Milli Parkı; 18.5 km from Abant lake
<i>A. campestre</i>	Denk & Grimm 2006088 S	ca40	Turkey; Bolu to Abant Gölu Milli Parkı; 18.5 km from Abant lake
<i>A. campestre</i>	Denk & Grimm 2006127 S	ca41	Turkey; SE of Zonguldak. valley of Yenice/Filyos river, 30 to 40 km before Yenice
<i>A. orthocampestre</i> sp. nov.	Denk & Grimm 2006183 ISTO/S	ca42	Turkey; Çehennem Deresi Kanyonü; small side gorge of the valley of the Berta Suyu, a tributary of the Çoruh River
<i>A. orthocampestre</i> sp. nov.	Denk 989110-12 ISTO/TBI	ca43	Georgia; Pichora River, behind Paleostomi (Paliostomi) Lake (42°8.5'N, 41°49.5'E), near sea level
<i>A. campestre</i>	Denk s.n., leg. 15/08/1995 S	ca44	Hungary; Lake Balaton
<i>A. campestre</i>	Denk 978020 S	ca45	Bulgaria; Loveç
<i>A. campestre</i>	Denk 978262 S	ca46	Bulgaria; Llulin Mountains
<i>A. orthocampestre</i> sp. nov.	Denk & Grimm 2006182 ISTO/S	ca47	Turkey; Çehennem Deresi Kanyonü; small side gorge of the valley of the Berta Suyu, a tributary of the Çoruh River
<i>A. campestre</i>	Denk & Grimm 2006160 S	ca48	Turkey; road from Samsun to Bafra
<i>A. campestre</i>	Schlee MS01408 S	ca49	Germany, Rheinland-Pfalz; Bad Kreuznach
<i>A. campestre</i>	Schlee MS01409 S	ca50	Switzerland, canton Basel-Land; in the vicinity of Basel
<i>A. campestre</i>	Grimm GG01411 (TUB)S	ca51	Germany, Rheinland-Pfalz; near Tawern
<i>A. campestre</i>	Grimm GG01412 (TUB)S	ca52	Germany, Rheinland-Pfalz; near Tawern-Fellerich, between Mosel and Saar valleys
<i>A. campestre</i>	Grimm GG01413 (TUB)S	ca53	Germany, Rheinland-Pfalz; near Tawern-Fellerich, between Mosel and Saar valleys
<i>A. campestre</i>	Grimm GG01415 (TUB)S	ca54	Germany, Baden-Württemberg; Goldersbachtal, near Bebenhausen
<i>A. campestre</i>	Grimm GG01416 (TUB)S	ca55	Germany, Baden-Württemberg; Goldersbachtal, near Bebenhausen
<i>A. campestre</i>	Denk & Grimm 2006035 S	ca56	Turkey; Longos forest S of Iğneada
<i>A. campestre</i>	Denk & Grimm 2006034 S	ca57	Turkey; Longos forest S of Iğneada
<i>A. campestre</i>	Denk & Grimm 2006033 S	ca58	Turkey; Longos forest S of Iğneada
<i>A. campestre</i>	Denk & Grimm 2006004 S	ca59	Turkey; road from Kemerburgaz to Kestanelik; W of Odayeri
<i>A. campestre</i>	Grimm GG01452 (TUB)S	ca60	Germany, Baden-Württemberg; Nord-Schwarzwald (northern part of Black Forest)

Table. (Continued).

Species	Voucher	Clone no.	Locality
<i>A. campestre</i>	Grimm GG01453 (TUB)S	ca61	Germany, Baden-Württemberg; Nord-Schwarzwald (northern part of Black Forest)
<i>A. campestre</i>	Denk & Tschan 2007113 S	ca62	Italy, Basilicata; between Senise and San Severino Lucano
<i>A. cappadocicum</i> subsp. <i>cappadocicum</i>	Langer BGT01020 TUB	cd00	Cultivated; arboretum, University of Tübingen
<i>A. cappadocicum</i> subsp. <i>cappadocicum</i>	Denk 896183 S	cd01	Georgia; Aragwi valley, Likoki gorge
<i>A. cappadocicum</i> subsp. <i>cappadocicum</i>	T. Denk, s.n., leg. 24/7/1996 S	cd02	Turkey; hinterland of Trabzon
<i>A. cappadocicum</i> subsp. <i>cappadocicum</i>	Denk & Grimm 2006177 S	cd03	Turkey; Çehennem Deresi Kanyonu; small side gorge of the valley of the Berta Suyu, a tributary of the Çoruh River
<i>A. cappadocicum</i> subsp. <i>divergens</i>	Denk & Grimm 2006178 S	dv01	Turkey; upper Çoruh valley (valley of the Berta Suyu tributary); along street from Artvin to Ardanuç
<i>A. cappadocicum</i> subsp. <i>divergens</i>	Denk & Grimm 2006180 S	dv03	Turkey; Upper Çoruh valley (valley of the Berta Suyu tributary); along street from Artvin to Ardanuç
<i>A. lobelii</i>	Denk & Tschan 2007021 S	lo01	Italy, Campania; between Serre and Polla, northern slopes of Monte Alburno
<i>A. lobelii</i>	Denk & Tschan 2007023 S	lo03	Italy, Campania; between Serre and Polla, northern slopes of Monte Alburno
<i>A. lobelii</i>	Denk & Tschan 2007025 S	lo04	Italy, Campania; between Serre and Polla, northern slopes of Monte Alburno
<i>A. lobelii</i>	Denk & Tschan 2007034 S	lo05	Italy, Basilicata; between Brienza and Pignola, "Fossa Cupa", NW of Monte Arioso
<i>A. lobelii</i>	Denk & Tschan 2007125 S	lo06	Italy, Basilicata; Parco Nazionale del Pollino, slope of Monte Pollino, ca. 10 km SSE of San Severino Lucano
<i>A. lobelii</i>	Denk & Tschan 2007126 S	lo07	Italy, Basilicata; Parco Nazionale del Pollino, slope of Monte Pollino, ca. 10 km SSE of San Severino Lucano
<i>A. lobelii</i>	Denk & Tschan 2007127 S	lo08	Italy, Basilicata; Parco Nazionale del Pollino, slope of Monte Pollino, ca. 10 km SSE of San Severino Lucano
<i>A. miyabei</i>	Edelmann MA01088 (TUB) S	my00	Cultivated; collected in Morris Arboretum, specimen no. (J17) 51-292*A
<i>A. okamotoanum</i>	Sun 4194 W	ok01	See Pfosser et al. (2002)
<i>A. platanoides</i>	Langer La01004 TUB	pl04	Germany, Baden-Württemberg; Königsfeld im Schwarzwald
<i>A. platanoides</i>	Schlee MS01128 (TUB)S	pl11	Hungary; Budapest, Gellért-hegy
<i>A. platanoides</i>	Schlee MS01138 (TUB) S	pl12	France, dépt. Alpes-de-Haute-Provence; Sisteron
<i>A. platanoides</i>	Denk 896167/D S	pl13	Georgia; Caucasus Mts
<i>A. platanoides</i>	Grimm & Blessing GG01159 (TUB) S	pl14	Sweden; Skeen, next to lake; 10 km W of Ljungby
<i>A. platanoides</i>	Grimm & Blessing GG01160-62 (TUB) S	pl15	Sweden; Skeen, next to lake; 10 km W of Ljungby; 3 individuals
<i>A. platanoides</i>	Denk & Grimm 2006098 S	pl17	Turkey; above Abant Gölü (lake); Abant Gölü Milli Parkı
<i>A. platanoides</i>	Grimm GG01418 S	pl18	Germany, Baden-Württemberg; Goldersbachtal, near Bebenhausen
<i>A. truncatum</i>	Langer BGT01060 TUB	tr00	Cultivated; arboretum, University of Tübingen

2.2. Molecular analyses

As far as new data are reported in this study (Table), DNA extraction, PCR, and cloning protocols are the same as in earlier studies on *Acer* (Grimm et al., 2006, 2007) following the specifications of Denk et al. (2002). Total DNA was extracted using a modified CTAB protocol (Gebhardt et al., 1989); PCR was done with the angiosperm-specific primers (Torres et al., 1990; Jobst et al., 1998). The ITS region of the nuclear-encoded 35S rDNA cistron comprising the internal transcribed spacers ITS1 and ITS2 and the 5.8S rDNA was amplified and cloned using a pUC18/DH5 α system. Up to 11 positive clones per individual were sequenced by a professional lab (LGC Genomics, Berlin). New sequences have been submitted to the European Nucleotide Archive and can be accessed via the following link: <http://www.ebi.ac.uk/ena/data/view/LK022453-LK022679>. The final matrix dimensions are 377 taxa (sequenced clones) and 787 characters (including 523 from the ITS1 and ITS2).

2.3. Phylogenetic analyses

Trees were inferred under the maximum likelihood criterion using the original matrix of clone sequences and a matrix of strict individual-consensus sequences using RAxML v. 7.2.6 (Stamatakis, 2006; Stamatakis et al., 2008) as the latest working version compiled for DOS/Windows. Bootstrap (BS) support was established using the fast bootstrap implementation in RAxML (Stamatakis et al., 2008); number of necessary bootstrap replicates were determined by the extended majority rule bootstrap criterion (Pattengale et al., 2009). The matrix of strict individual-consensus sequences was (a) analysed in the standard setting (GTR-A) using a 4 \times 4 general-time reversible substitution model (GTR; Rodriguez et al., 1990) allowing for site-rate variation modelled via a Gamma distribution (+ Γ), followed by (b) treating all nucleotides and polymorphisms as distinct characters (Potts et al., 2014) using the multistate model implemented in RAxML (-m MULTIGAMMA). Two models were applied for (b): a GTR model allowing for unequal substitution rates between all possible states (GTR-I) and Lewis' (2001) Markov-model, which optimises a single substitution probability (MK-I).

In addition to ML-inferred trees and BS branch support, we computed planar phylogenetic networks, neighbour-net splits graphs (NNet; Bryant and Moulton, 2002, 2004), based on pairwise interclone and interindividual distance matrices using SplitsTree v. 4.13 (Huson and Bryant, 2006). Interclone and interindividual distances were computed with PAUP* (Swofford, 2002) using model-based distances based on the model parameters optimised by RAxML for the clone data set. Interindividual distances were computed with the programme PBC using the phylogenetic Bray–Curtis distance transformation (PBC;

Göker and Grimm, 2008). The PBC transformation performed best out of several tested transformations for 4 plant data sets including *Acer* section *Acer* (Göker and Grimm, 2008).

Branch-support from the ML bootstrapping was mapped onto the NNet for corresponding edges by investigating the bootstrap sample using bipartition networks, a special form of consensus networks (Holland and Moulton, 2003; Grimm et al., 2006).

All input files (character and distance matrices) and output files (trees, bootstrap samples, networks, etc.) are included in the electronic supplementary archive (ESA) to this article (www.palaeogrimm.org/data/GD14TJB_ESA.zip).

2.4. Detailed sequence analyses of genotypes defining cryptic species in *A. campestre*

We visually analysed sequence motives in 2 ITS regions with length-polymorphism that are diagnostic at species to section levels in *Acer* (Grimm, 2003; Grimm et al., 2007). This was done for all species of section *Platanoidea* and its putative sister clades (*A. macrophyllum*, series *Macrophylla*; section *Macrantha*). This allows illustrating the putative ancestry of the Colchic genotype of *A. campestre* in relation to the main-genotype *A. campestre* (see Section 3).

Furthermore, based on the clone sequence alignment, we filtered all positions that are variable between (and within) main-genotype *A. campestre* individuals throughout their distribution range. The data, 4 single-nucleotide site variations and 2 length-polymorphisms in ITS1 and 3 single-nucleotide site variations in ITS2, were tabulated (File S1 in ESA) and placed into an evolutionary context using the principles of median/median-joining networks (Bandelt et al., 1995, 1999) following the guidelines of Bandelt et al. (2000).

3. Results

3.1. General ITS differentiation and phylogenetic relationships

Interclone distances reveal the distinctness of the ITS genotypes of the 3 Colchic *A. campestre* individuals. The overall genetic divergence between the Colchic and the main genotype of *A. campestre* equals divergence encountered between species of section *Platanoidea*. Clones of *A. cappadocicum* are most distinct (Figure 1). This distinctness causes a split in the clone assemblage of *A. campestre* in an according maximum likelihood tree (Figure 2): clones from the Colchic individuals are placed in between the *A. cappadocicum* species complex subtree and the subtree including the remainder of section *Platanoidea*. Moreover, the root-tip distance is relatively low. This is a first hint that the Colchic genotype of *A. campestre* represents an archaic ITS variant of section

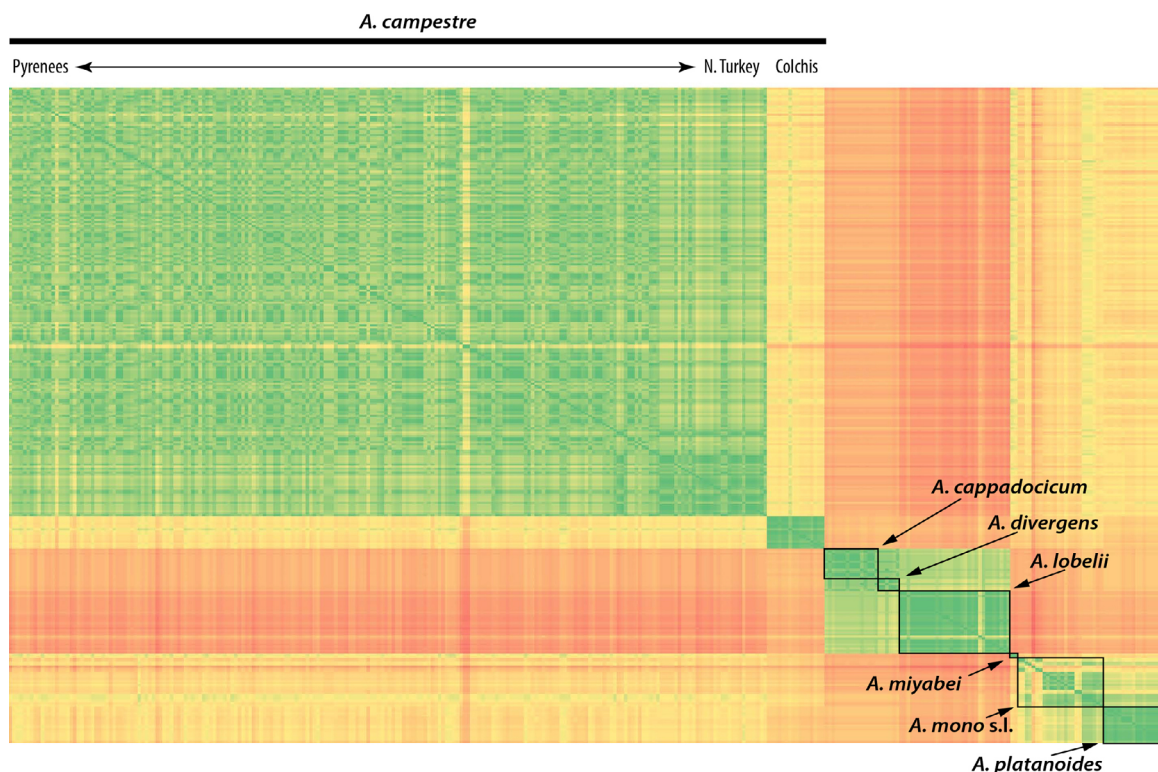


Figure 1. Heat-map based on pairwise model-based interclone distances. Distinct *Acer campestre* clones from the Colchic region are readily visible (pseudogenous clones not included).

Platanoidea. Two markedly pseudogenous ITS clones obtained from Bulgarian individuals of *A. campestre* nest within the Colchic *A. campestre* and the *A. cappadocicum* species complex.

The individual-based analyses show that the individual of *A. miyabei* is the closest extant relative of *A. campestre* individuals with the main genotype (Figure 3). The Colchic individuals are placed between the *A. cappadocicum* species complex and main-type *A. campestre* (+ *A. miyabei*), essentially forming an evolutionary link between these 2 lineages. The phylogenetic signal from the Colchic *A. campestre* is highly ambiguous, which explains the low bootstrap support along the backbone branches of the clone-based ML tree: tree inference, limited to dichotomy, has to decide for one alternative (Figure 2), even if there is a strong conflicting signal in the underlying data. The topological alternative for *Acer* section *Platanoidea* is that all *A. campestre* share a common origin, as it would be naturally assumed (Figure 3).

3.2. Diagnostic value of ITS sequences of *A. campestre*

Three of the length polymorphic regions (LP), which are known to provide diagnostic motives at and above the species level in genus *Acer* (LP1 to LP3; Grimm, 2003; Grimm et al., 2006, 2007), demonstrate the close

relationship between both genotypes of *A. campestre*, and *A. miyabei* to a certain degree (Figures 4 and 5). For example, most ITS clones of main-type *A. campestre* and both clones of *A. miyabei* lack the initial A in the LP1, a common and conserved feature of section *Platanoidea*. Two sequence characteristics that are restricted to widespread ITS variants of main-type *A. campestre* are missing in the Colchic genotype. In contrast, the LP3 illustrates the common origin of both *A. campestre* genotypes.

3.3. ITS differentiation pathways in *A. campestre*

From the alignment, 9 variable sequence patterns (6 from ITS1, 3 from ITS2) can be extracted that contribute to the increased intra- and interindividual variability in main-type *A. campestre* (File S1 in ESA). The splits promoted by these positions are to a large degree incompatible, but can be arranged in a mutational framework using the principles behind median networks and a threshold of 2 required differences to define general ITS variants (Figures S1–S3 in ESA). Turkish and East Bulgarian main-type individuals exhibit up to 3 ITS variants, of which 1 (orange variant in Figure 6) is more closely related to the Colchic genotype (red variant) than others. In the western part of the distribution area of *A. campestre* (Central Bulgaria to North Spain), the orange variant is replaced

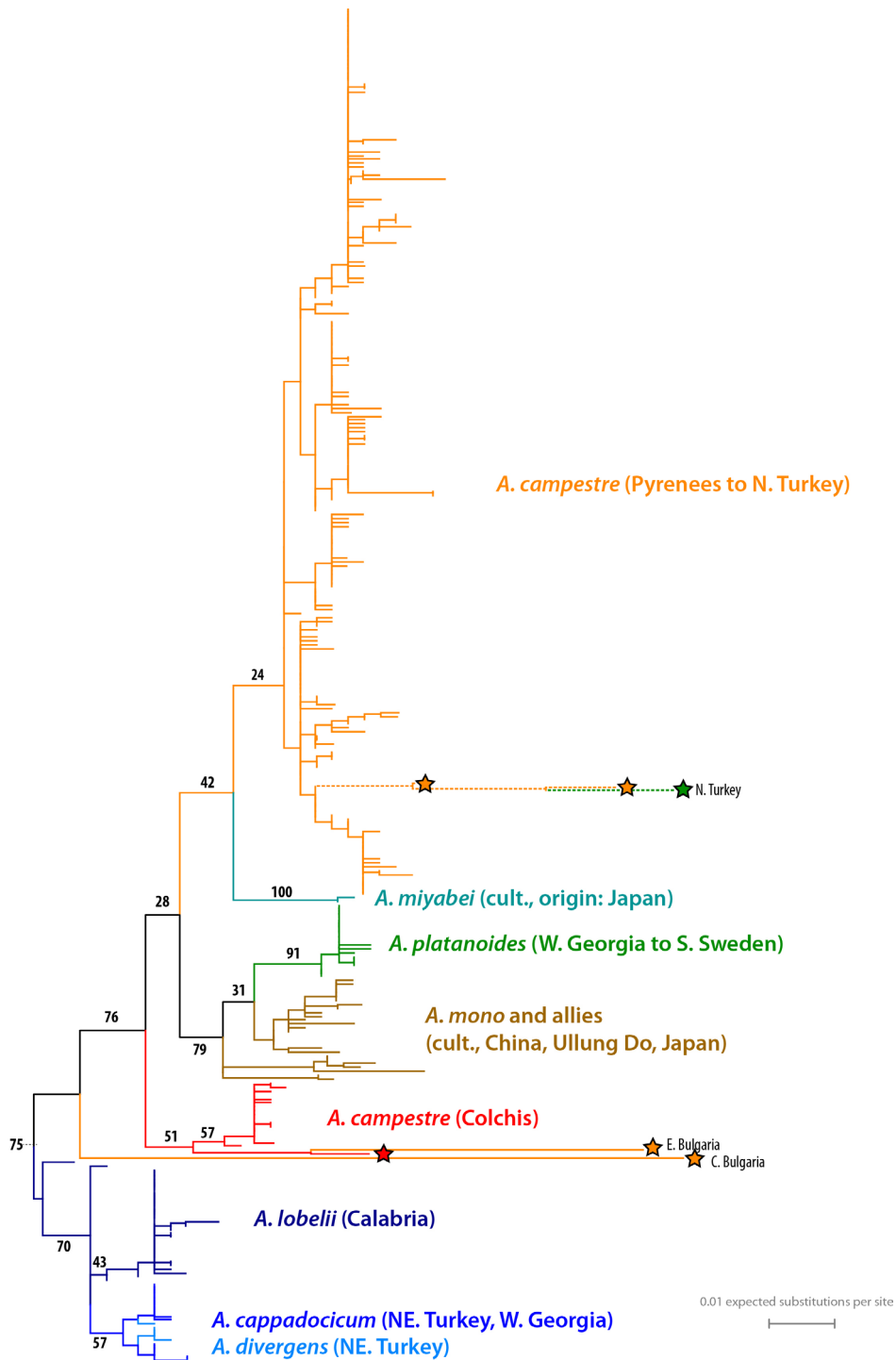


Figure 2. Comprehensive maximum likelihood (ML) tree based on 344 ITS clones of *Acer campestre* and other species of section *Platanoidea*. The tree has been rooted under the assumption that the *Acer cappadocicum* complex represents the earliest diverging lineage within the section *Platanoidea* (Grimm et al., 2006). Very long terminal branches indicate clones with (partially) pseudogenous sequences. Numbers at selected branches show bootstrap support based on 1000 ML bootstrap replicates. Stippled lines: branch length reduced to one-third.

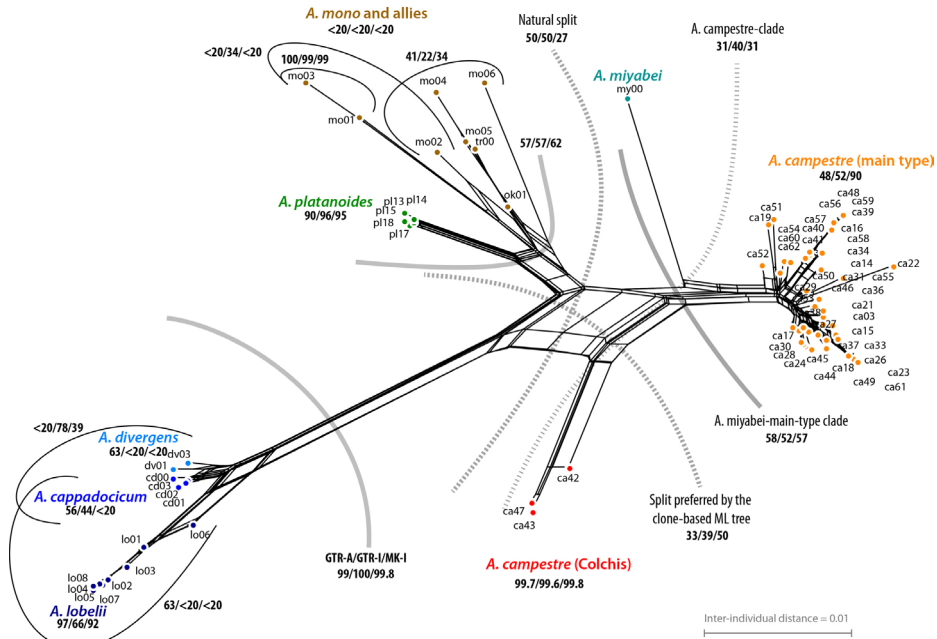


Figure 3. Neighbour-net splits graph based on interindividual distances. Numbers refer to support of the according edge under maximum likelihood using different transition models (GTR-A, GTR-I, MK-I: see Section 2).

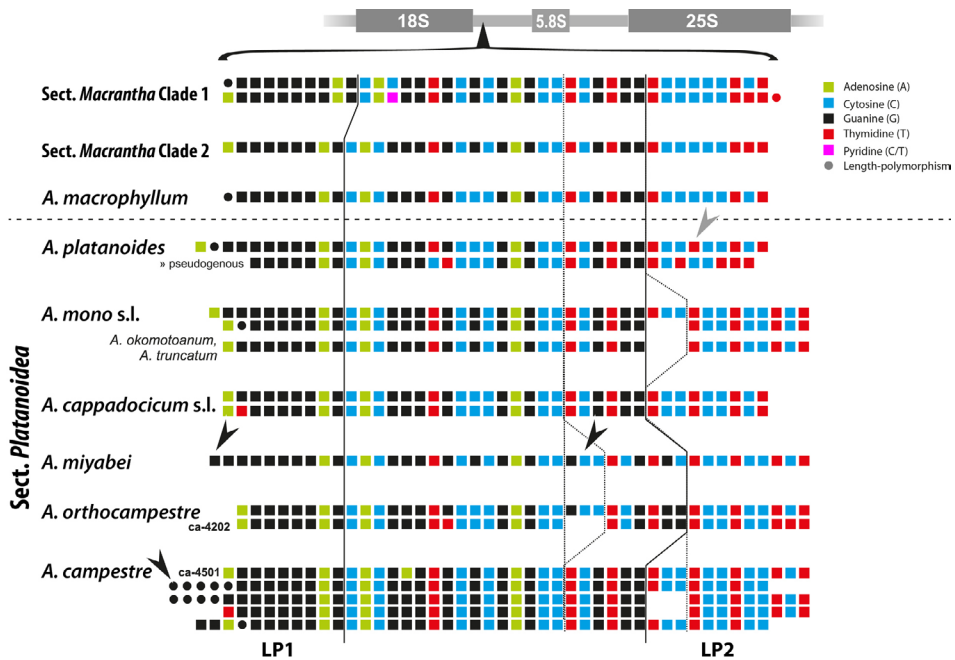


Figure 4. Part of the ITS1 of section *Platanoidea* and its putative sister clades (*A. macrophyllum*, section *Macrantha*; Grimm et al., 2006; Renner et al., 2008), including species- and clade-diagnostic length-polymorphic motives LP1 and LP2. The LP1 is relatively conserved among the taxa of section *Platanoidea*. Typically an initial adenosine (A) is followed by 6-8 guanines (G) in section *Platanoidea* except for most *A. campestre* and *A. miyabei*. Downstream, within the generally length-conserved part, a GCC duplication is exclusively found in *A. miyabei* and the Colchic *A. campestre*, whereas the terminal pyrimidine-dominated LP2 motif has 3 subtypes not exclusively restricted to a single species. Within *A. campestre* 4 basic sequence types can be recognised, differing in the 5' guanine-dominated LP1 motif. The Colchic *A. campestre* has a LP1 motif with initial A, missing in most main-type *A. campestre* and *A. miyabei* (as in most other *Acer* spp.) In Central and South France, another motif type is found with an initial thymidine (T) followed by 6 G. A third type (GGA-6G) is exclusively found in individuals from North Spain and South-East France.

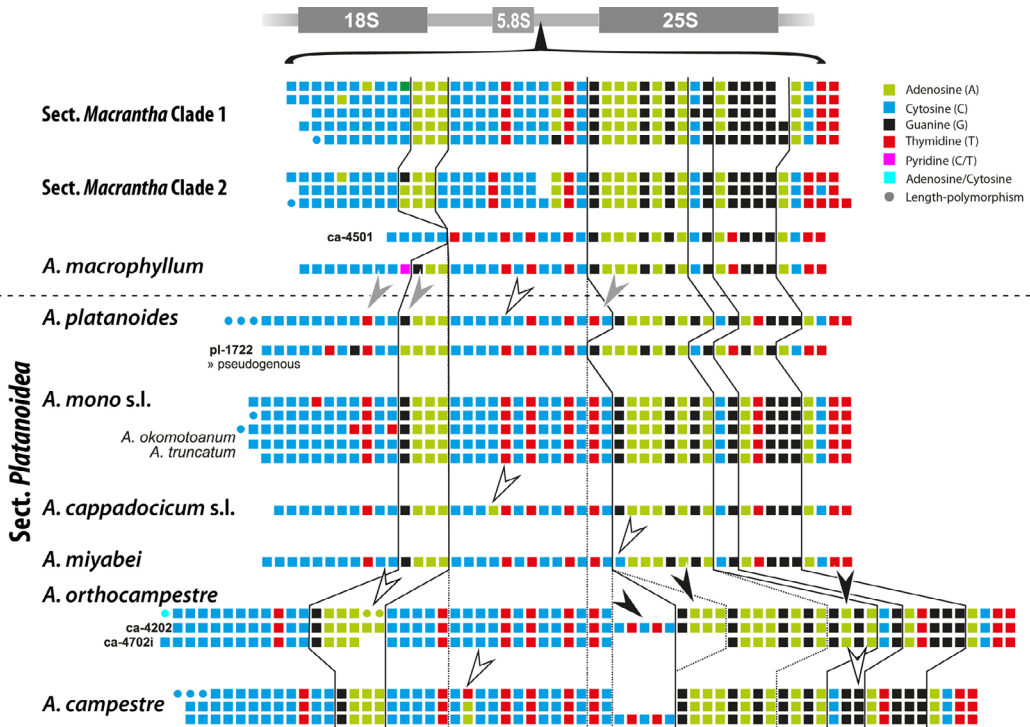


Figure 5. LP3 sequence types in section *Platanoidea* and its putative sister clades. The sequence of the LP3 is highly diagnostic for section *Platanoidea* (GAAA after the initial C-dominated motif, TC duplication; grey arrows). Single, conserved sites differentiate between the species/species complexes of the section (white arrows). The central pyrimidine-purine motives reflect the common origin of both *A. campestre* genotype (Colchic and main-type; black arrows). A compensatory duplication feature distinguishes *A. campestre* from the other taxa comprising 5 + 4 nt in the central and terminal part. A downstream-located double G instead a single G exclusively found in main-type *A. campestre* and no other *Acer* spp. differentiates between the 2 genotypes.

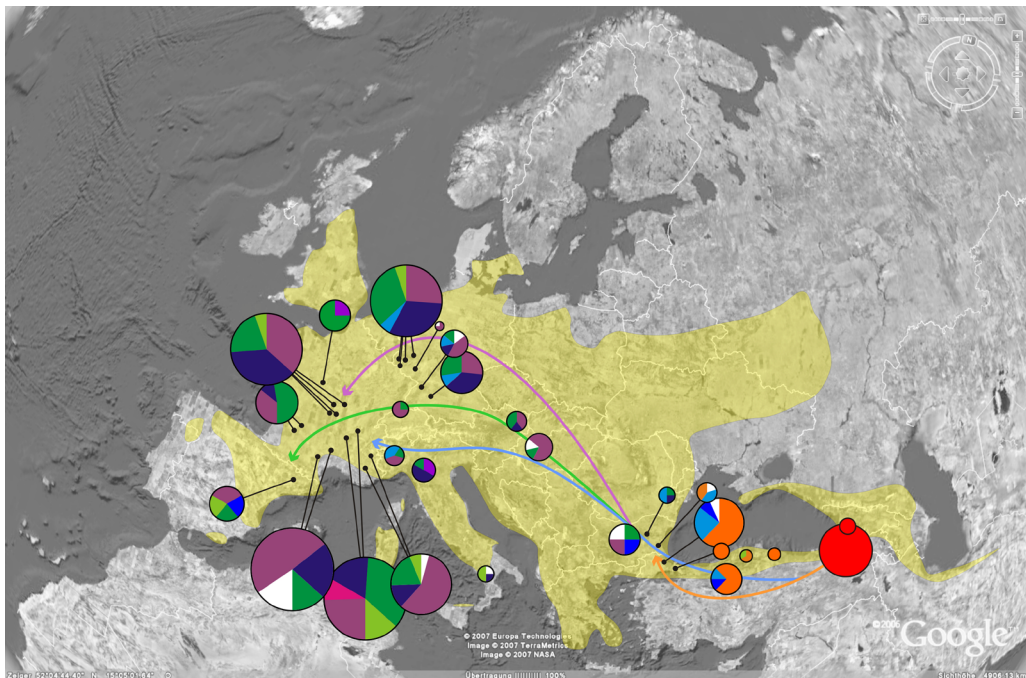


Figure 6. Distribution of major ITS variants (File S1; Figures S1–3 in ESA) of *Acer campestre* in West Eurasia. Red, Colchic genotype; all other colours, main-type. Coloured lines refer to the putative evolution pathway of ITS variants and their subsequent dispersal through the gene pool of main-type *A. campestre*.

by increasingly derived ITS variants (green, blue, purple). Two of these (light and dark blue) are also encountered in the Turkish and East Bulgarian individuals.

3.4. *Acer orthocampestre* G.Grimm & Denk sp. nov. (Figures 7 and 8)

Type (Figure 7): Georgia: Pichora River, behind Paleostomi (Paliostomi) Lake (42°8.5'N, 41°49.5'E), near sea level, liana-rich natural levee-forest, close to or even in the water (Denk et al., 2001, locality 4), 21/9/1998, T. Denk 989110–12 (holotype: ISTO, isotype: TBI).

Additional material (paratypes; Fig. 8): Turkey, A9, Artvin, Ardanuç Belediyesi, Çehennem Deresi Kanyonu (Devil-Torrent Canyon; 41°8'N, 42°3'E), small side gorge into the valley of the Berta Suyu, a tributary of the Çoruh, 2/8/2006, T. Denk & G. Grimm 2006-182 ISTO/S and 2006-183 ISTO/S.

Diagnosis: *Acer orthocampestre* differs from all other species of *Acer* by a unique ITS sequence representing a putative ancient sequence type of the *A. campestre*-(*A. miyabei*) lineage within section *Platanoidea* (ENA accession numbers LK022605–LK022622). It differs from



Figure 7. *Acer orthocampestre* sp. nov., holotype.



Figure 8. *Acer orthocampestre* sp. nov., paratype.

the ITS of *Acer campestre* at positions 77, 79, LP1 (pos. 111–121), pos. 140–142 (3-nt long duplication, upstream LP2), 163, 166, 251, 278, 285 in the ITS1 and at positions 509, 543–544 (within LP3), 635, 637 in ITS2 (reference alignment included in ESA).

Description: Morphologically (habit, leaves, samaras) *Acer orthocampestre* cannot be distinguished from *A. campestre*. Preliminary data indicate that the 5S intergenic spacer regions also strictly discriminate between the *A. orthocampestre* with Colchic ITS genotypes and the main-

type *A. campestre* (R Volkov and co-workers, unpublished data). No flowering material has been studied so far.

Ecology: *Acer orthocampestre* grows in temporally flooded riparian stands (Denk et al., 2001) and gorges/small canyons of the Colchic region (North-East Turkey, West Georgia). The Georgian stand is characterised by a fully humid warm-temperate *Cfa* climate, with no dry season. The regional climate in North-East Turkey is a *Cfb/Dfb* climate. The canyon of the Berta Suyu, however, is characterised by strong seasonality. Typical tree species

in the main canyon are *Acer (cappadocicum* subsp.) *divergens*, *Platanus orientalis* L. associated with *Celtis australis* L., *C. caucasica* Willd. (treated as a subspecies of *C. australis* by some authors), *Ficus carica* L., and *Juglans regia* L., giving the riparian flora in the main canyon a sub-Mediterranean appearance. The fully humid character of the region is underpinned by the occurrence of *Asplenium scolopendrium* L. and *Diospyros lotus* L. The Çehennem gorge itself supports a moist and mild microclimate also during August, when the material was collected. Associated with several individuals of *Acer orthocampestre* were large-leaved *A. cappadocicum*, morphologically (and genetically) distinct from the *A. divergens* in the main gorge.

Etymology: The specific epithet conveys that the Colchic populations previously included within *A. campestre* based on indistinct morphology likely represent survivors of the evolutionary lineage from which the West Eurasian *A. campestre* and possibly also the East Asian *A. miyabei* are derived.

Distribution: The newly recognised species is known from 2 stands, c. 100 km air-distance apart. On-going research with a focus on East European *A. campestre* including material from the disjunct Ukrainian populations (West Ukraine, Crimean peninsula, and material covered by this study) indicate that to the north-west *A. orthocampestre* occurs at least until the Crimean peninsula (R Volkov and co-workers, unpublished data). No *A. campestre* genotypes have been found so far south-east of the Don. There is apparently a distribution gap between the area west and north of the Don delta (*A. campestre*) and the north-west extension of the Colchic populations in the south part of the Crimean peninsula (*A. orthocampestre*). Its distribution towards the east is unknown. However, based on the distributional pattern of *A. campestre* in the North Caucasus, East Georgia, Azerbaijan, and North Iran, where it is confined to *Cfa*, *Cfb* climates, following strictly the river valleys, we assume that all (natural) populations of *A. campestre* east of the verified occurrences might belong to *A. orthocampestre*.

4. Discussion

4.1. *Acer orthocampestre* sp. nov., a rare example of true cryptic speciation in flowering plants

As a rule, morphological differentiation often outruns genetic differentiation in northern hemispheric tree genera, at least when genetic differentiation is studied using molecular sequences and a comprehensive sampling (more than one individual per species). Striking examples are the northern hemispheric white oaks (*Quercus* Group *Quercus*), where identical ITS variants are found in species of West Eurasia, North America, and East Asia (Denk and Grimm, 2010) and disjunct sister species in beech trees (Denk et al., 2005): *Fagus sylvatica* (West

Eurasia)–*F. crenata* (Japan); *F. engleriana* (mainland China)–*F. japonica* (Japan). Nevertheless, in the genus *Acer*, broad-sampled ITS data discriminate between closely related species of section *Acer* (Grimm et al., 2007). The same may hold true for most other sections (Grimm et al., 2006), although comprehensive sampling at and below the species level is still lacking for most lineages. Plastid data, stored in gene banks, do not currently provide an alternative for genotaxonomy at the species level, because of incomplete sampling (except for the species pair *A. saccharinum*–*A. rubrum*) and generally lower divergence (G. Grimm, pers. obs.; gene bank accessed 24/3/2014; File S2 in ESA). In section *Platanoidea*, ITS data clearly distinguish *A. platanooides*, *A. lobelii*, and *A. miyabei* (Figures 1–3). Patterns of intraindividual ITS variability and detailed sequence analysis (Figure 6; File S1 in ES) further allow distinguishing between *A. cappadocicum* and *A. divergens* individuals growing in close vicinity. The situation in the *A. pictum* complex (*A. mono*, *A. truncatum*, *A. okamotoanum*) appears to be more complicated and requires a much more comprehensive sampling before any conclusions can be drawn. In light of the documented overall interspecies ITS divergence in section *Platanoidea* (this study), section *Acer* (Grimm et al., 2007), and the genus in general (Grimm, 2003; Grimm et al., 2006), the 2 genotypes of *A. campestre* (Figures 1–5) warrant recognition as 2 distinct species (see Mallet, 2007, p. 279). Since *Acer campestre* is widely cultivated and shows a continuous distribution across the Black Sea region of Turkey into adjacent Georgia and Armenia (Figure 6), one could expect individuals with mixed genotypes (main-type and Colchic type). However, this is not the case.

Therefore, we formally describe a new species, *A. orthocampestre*, to accommodate the current (and future) individuals of *A. campestre* showing the Colchic ITS genotype. The new species is a true cryptic species as it lacks any morphological feature that distinguishes it from *A. campestre* s.str. (see Section 3.4)

Cryptic speciation is common in organisms with limited morphological features such as protozoans (Weiner et al., 2014), fungi (Göker et al., 2009), lichen, bryophytes (Draper et al., 2007), and pteridophytes (Yatabe and Murakami, 2003). Recently, cryptic species have also been reported in higher plants (Abdelaziz et al., 2011), including studies backed by crossing experiments (Okuyama and Kato, 2009). However, most reported cases of cryptic species in flowering plants may actually represent pseudocryptic speciation: genotaxonomy identifies hitherto overlooked morphological entities. A further candidate for pseudocryptic speciation in West Eurasia is *Fagus sylvatica* s.l. (Denk, 1999a, 1999b; Denk et al., 2002; Gömöry and Paule, 2010) and *Acer monspessulanum* (Grimm et al., 2007).

In contrast, the Colchic field maple populations of *A. orthocampstre* entirely fall within the morphological variation of *A. campestre* as illustrated in Krüssmann (1960), verified by our own collection and field experience. Specimens characterised by main-type ITS variants include leaf morphotypes indistinguishable from the genotyped Colchic samples. Furthermore, there is similarity between the here-designated holotype and isotype of *A. orthocampstre* (Figures 7 and 8) and the Linnean holotype of *A. campestre* (<http://linnean-online.org/12349/>).

4.2. The Colchic region, archive of ancient plant lineages

The potential of ITS to reconstruct complex processes of intrageneric evolution, partly including information from pseudogenous variants (Razafimandimbison et al., 2004; Grimm and Denk, 2008), has been demonstrated in many cases. Studies with a comparable sampling and geographic cover are available for *Acer* section *Acer* (Grimm et al., 2007), *Fagus* (Denk et al., 2002, 2005), *Quercus* (Denk and Grimm, 2010), and *Zelkova* (Denk and Grimm, 2005; Christie et al., 2014). In *Fagus*, *Quercus* Group Ilex, and *Zelkova*, species restricted to West Eurasia show close links to the Himalayan region and into East Asia. For instance, the Mediterranean sclerophyllous oaks (3 species) belong to a wider group of oaks (*Quercus* Group Ilex) with a distribution from the Hindu Kush along the south flank of the Himalayas into China and Japan (Denk and Grimm, 2010).

The closest relative of the West Eurasian *Fagus sylvatica* L. (s.l.), based on ITS data and morphology, is the Japanese *F. crenata* Blume (Shen, 1992; Denk, 2003; Denk et al., 2005). In *F. sylvatica* s.l. the Caucasian and North-East Turkish populations are genetically and morphologically distinct from the populations in the west parts of Turkey and throughout Europe (Denk, 1999a, 1999b; Gömöry and Paule, 2010). Most notably, a morphological border can be drawn between the lowland Longos populations of South-East Bulgaria/North-West Turkey (cupules with green leaflets) and the mountain populations in Central/West Bulgaria (and the rest of Europe, cupules never with green leaflets) that corresponds to a genocline in the same area, a gradual change in genetic signatures from North Turkey to Central, North, and West Europe (Gömöry and Paule, 2010). This pattern is somewhat analogous to the situation in (main-type) *A. campestre* with the 'orange' ITS variant (Figure 6) being restricted to South-East Bulgaria and (West) Turkey, but co-occurring with (relatively underived) variants found also in the remainder of the range of main-type *A. campestre*. The only difference is that in *Fagus sylvatica* (s.l.) morphology draws a clear line, whereas in *A. campestre* (s.str., main genotype), no morphological difference has been observed so far.

In *Zelkova*, the Caucasian-North Iranian species *Z. carpinifolia* (Pall.) C.Koch forms the phylogenetic bridge between the highly endemic disjunct sister species *Z. abelicea* (Lam.) Boiss. (Crete) and *Z. sicula* DiPasq., Garfi & Quezel (Sicily), and *Z. serrata* (Thunb.) Makino with a distribution from Central and North China into Japan (Denk and Grimm, 2005). Detailed analyses of West Eurasian populations of *Zelkova* confirmed this pattern and the uniqueness of the Caucasian populations of *Z. carpinifolia* using a much broader sample (Christie et al., 2014). Additionally, in *Acer* section *Acer* a (relatively deep) phylogenetic split can be found between the Colchic taxa and their western counterparts. *Acer ibericum* M.Bieb. ex Willd., with a possible similar range to *A. orthocampstre* (Colchic genotype), has been variously treated as a subspecies of *A. monspessulanum* L., a species widespread in the West Mediterranean region extending into Turkey and the Levant. However, ITS variants of *A. monspessulanum* and *A. ibericum* are highly distinct, the latter being genetically closer related to the morphologically much more distinct *A. hyrcanum* Fisch. & C.A.Mey. (ranging from the Balkans into Iran). The same differentiation pattern is also seen in preliminary 5S IGS data of the *A. monspessulanum* species complex covering all eastern subspecies/variants assigned to this species (unpublished data). This highlights the importance of the Colchic region – North-East Turkey and West Georgia – and adjacent regions as a repository for unique genotypes and a refuge of unique tree species.

4.3. Genetic signature of Neogene plant distribution patterns

The Neogene, the period between 23 and 2.6 million years ago, is characterised by a fairly homogeneous northern temperate vegetation (Mai, 1995; Manchester, 1999). For example, plant taxa today restricted to western North America (*Sequoia*) or to East Asia (*Glyptostrobus*) had a wide distribution in the northern hemisphere including modern-day Turkey and Greece (Güner and Denk, 2012; Velitzelos et al., 2014). The disruption of formerly connected biota happened in the middle Miocene and was linked to the uplift of the Himalayas and the formation of the Tibetan Plateau (Ramstein et al., 1997). Following the disjunction, speciation occurred in many temperate plant genera. Prior to the isolation of the 2 areas (West Eurasia, East Asia), morphotypes with strong similarity to modern East and South-East Asian species were common in the Euro-Mediterranean region, such as *Mahonia* Group Orientales (Güner and Denk, 2012), *Berberis* Group Australes (Kvaček and Erdei, 2001), and *Quercus* Group Ilex (Velitzelos et al., 2014). After the isolation, East Asian morphotypes gradually disappeared and were replaced by

modern West Eurasian morphotypes. This is exemplarily illustrated by the replacement of the Oligo-Miocene *Fagus castaneifolia* Unger (North American-East Asian affinities) with the Mio-Pliocene *F. haidingeri* Kováts (East Asian-European affinities) and finally the modern *F. sylvatica* (Denk, 2004; Denk and Grimm, 2009). This morphological transition is imprinted in the gene pool of the modern West Eurasia *Fagus sylvatica* species complex, which has an ITS signature that is part of the natural variability of its East Asian counterparts (Denk et al., 2005). Morphotypes with clear similarities to *Acer (ortho)campestre* appear not prior to the late Miocene based on the fossil record of Europe (Walther, 1972). However, in East Asia, morphotypes comparable to *A. (ortho)campestre* and *A. miyabei* can possibly be traced back to the Paleogene (*A. kushiroanum* Tanai) and the early Neogene (*A. protomiyabei* Endo; Tanai, 1983). On the other hand, the *Acer cappadocicum* lineage, the first diverging lineage in section *Platanoidea* based on molecular data, can be traced back at least to the early Miocene of East Asia and Europe (Walter, 1973; Tanai, 1983). The recurrent pattern of genetically distinct and morphologically similar or identical taxon pairs in West Eurasia–East Asia, or (West) Mediterranean–Caucasia–East Asia, mirrors the historical biogeography of these lineages: unhindered gene flow and migration until the second half of the Neogene, and isolation and lineage sorting coupled with morphological stasis within the last ~15 million years.

5. Conclusion and outlook

In a number of widespread tree species and species complexes in West Eurasia (*A. campestre*-*A. orthocampestre*, *Acer monspessulanum* s.l., *Fagus sylvatica* s.l., *Zelkova* spp.) a clear genetic split between Colchic populations and their western counterparts is encountered. This calls for comprehensive taxonomic reinvestigations backed up by genetic data. Lumping taxa into a single, widespread species based on poorly understood morphological gradients as done for *Acer cappadocicum* (Murray, 1978, 1982), *A. monspessulanum* (Yaltirik, 1967), and *Fagus sylvatica* (Greuter and Burdet, 1981) may overlook important, genetically well-distinct species. This has implications for the assessment of biodiversity at regional and continental scales and on phylogenetic inferences at the genus and family levels. For a proper assessment the Euxinian-Caucasian-Hyrcanian region will need to be excessively sampled as many lineages in this region likely represent phylogenetic links between currently disjunct taxa in East Asia and Europe.

Ideally, species ought not be erected or rejected solely on the basis of descriptive (nonnumerical) morphology; any taxonomic reassessment should be complemented by genetic evidence (diagnostic sequences, DNA

fingerprinting, population-genetic analyses). The newly described true cryptic species *A. orthocampestre* is a special, and probably rare, case. Usually, distinct genetic signatures will go along with some sort of morphological or ecological differentiation (e.g., Borsch et al., 2011; Schlee et al., 2011). However, genotaxonomy should not generally replace morphology-based taxonomy. Recognising a new species because it differs by 1 or 2 nucleotides in a generally variable gene region without assessing the overall genetic variation in the master population and closely related species would be equally premature.

The recognition of the Colchic genotype as a species of its own right is a first step. The entire range of the new species within the morphospecies *A. campestre* remains to be clarified. For the Ukraine a study using PCR-RFLP and an additional marker, the 5S rDNA intergenic spacer, is in progress (R Volkov and co-workers, unpublished data). However, dense sampling in the Euxinian region of North Turkey would be particularly important in order to conserve the unique genetic resource of *Acer orthocampestre*. Furthermore, North Turkey may be the only place where both species come into direct (natural) contact and heterozygotes (hybrids) may occur. A further unresolved and important issue is the eastward extension of *A. orthocampestre* and its ecological characterisation. Finally, species closely resembling *A. campestre* in East Asia such as *A. miyabei* need to be studied in a similar way.

Regarding future phylogenetic studies on section *Platanoidea* or the genus *Acer* in general, the inclusion of material of *A. orthocampestre* and *A. campestre* is strongly recommended. The new cryptic species has a much less derived ITS genotype. It appears to be closer to the common ancestor of the whole section *Platanoidea* than other extant species. Hence, signals from ancient radiations may have been preserved in *A. orthocampestre*, but lost in more derived taxa. It would be most interesting to see which signal can be obtained from additional markers, in particular variable plastid regions.

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Content of the Electronic Supplementary Archive

File S1. Excel file including the following data: tabulation of variable positions in *A. campestre* (sheet 'ca-varpos'). Only such sites are shown that vary within the main-type *A. campestre*; site variation limited to a single clone is not shown.

File S2. Current sequence data coverage for the genus *Acer* (NCBI GenBank harvest, 24/3/2014).

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