Molecular Phylogenetics and Evolution 74 (2014) 97–110

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Disentangling relationships among the diploid members of the intricate genus *Knautia* (Caprifoliaceae, Dipsacoideae)

Ivana Rešetnik [a,1], Božo Frajman [b,1], Sandro Bogdanović [c], Friedrich Ehrendorfer [d], Peter Schönswetter [b,*]

[a] Faculty of Science, University of Zagreb, Marulićev trg 9a, HR-10000 Zagreb, Croatia
[b] Institute of Botany, University of Innsbruck, Sternwartestraße 15, A-6020 Innsbruck, Austria
[c] Faculty of Agriculture, University of Zagreb, Svetošimunska 25, HR-10000 Zagreb, Croatia
[d] Department of Botany and Biodiversity Research, University of Vienna, Rennweg 14, A-1030 Vienna, Austria

ARTICLE INFO

Article history:
Received 1 October 2013
Revised 19 January 2014
Accepted 27 January 2014
Available online 6 February 2014

Keywords:
Caprifoliaceae
Knautia
Annuals versus perennials
Haplotype sharing
Hybridisation
Polyploidy

ABSTRACT

The genus *Knautia* (Caprifoliaceae, Dipsacoideae) encompasses 40–60 species mainly distributed in western Eurasia, with highest species diversity in the Alps and the Balkan Peninsula. It is traditionally regarded as one of the taxonomically most challenging European genera due to the widespread occurrence of polyploidy, the high incidence of hybridisation and the maintenance of morphologically intermediate forms. A prerequisite for assessing the complex spatiotemporal diversification of a polyploid group is a comprehensive hypothesis of the phylogenetic relationships among its diploid members. To this end, DNA sequence data (nrDNA ITS and plastid *pet*N(*ycf*6)-*psb*M) combined with AFLP fingerprinting were performed on 148 diploid populations belonging to 35 taxa. Phylogenies obtained by maximum parsimony and Bayesian analyses were used to test the monophyly of the genus and its three sections *Trichera*, *Tricheroides* and *Knautia*, to provide insights into its evolutionary history and to test previous hypotheses of inter- and intrasectional classification. Both nuclear and chloroplast datasets support the monophyly of *Knautia* and its three sections, with ambiguous placement of *K.* cf. *degenii*. The majority of species belong to the nearly exclusively perennial section *Trichera* ($x = 10$). Within section *Trichera* all markers revealed largely unresolved phylogenetic relationships suggesting rapid radiation and recent range expansion. In addition, extensive sharing of plastid haplotypes across taxa and wide geographic ranges of plastid haplotypes and ribotype groups were observed. The molecular data are partly at odds with the traditional informal grouping of taxa within section *Trichera*. Whereas the traditional groups of *K. dinarica*, *K. drymeia* and *K. montana* can be maintained, the new, smaller and well supported Midzorensis and Pancicii Groups as well as the SW European Group are separated from the heterogeneous traditional *K. longifolia* group. The former groups of *K. arvensis*, *K. dalmatica*, *K. fleischmannii* and *K. velutina* are clearly polyphyletic. Their diploid members have to be rearranged into the Xerophytic Group, the Carinthiaca Group, and the Northern and Southern Arvensis Groups. The annual sections *Tricheroides* ($x = 10$) and *Knautia* ($x = 8$) with only a few taxa are resolved in the ITS and plastid trees on long branches as early diverging lineages within the genus.

1. Introduction

Polyploidisation, i.e. the multiplication of whole genomes, is among the most important mechanisms in plant speciation and accounts for ca. 15% of speciation events in vascular plant species (Wood et al., 2009). Polyploid complexes pose serious challenges to systematists and it is evident that only detailed knowledge of phylogenetic relationships among the sometimes geographically restricted and rare diploid members will allow understanding the evolution of their often far more widespread and abundant polyploid offspring.

A genus that has always been considered taxonomically difficult due to the frequent occurrence of polyploidy and the high incidence of hybridisation blurring species boundaries is *Knautia* L. (Caprifoliaceae, Dipsacoideae; Ehrendorfer, 1962a,b, 1981). Being the single member of tribe Knautieae within subfamily Dipsacoideae (Mayer and Ehrendorfer, 2013), it comprises 40–60 species and is distributed in western Eurasia and northwestern Africa with

* Corresponding author. Fax: +43 51250751099.
E-mail addresses: ivana.resetnik@biol.pmf.hr (I. Rešetnik), bozo.frajman@uibk.ac.at (B. Frajman), sbogdanovic@agr.hr (S. Bogdanović), friedrich.ehrendorfer@univie.ac.at (F. Ehrendorfer), peter.schoenswetter@uibk.ac.at (P. Schönswetter).
[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.ympev.2014.01.028
1055-7903/

highest species diversity in southern and southeastern Europe, especially the Alps and the Balkan Peninsula. Most species are perennial herbs with undivided to pinnate opposite leaves and hermaphrodite or female flowers (occasional gynodioecy occurs) arranged in capitula. The fruits bear a soft, lipid-rich elaiosome at their base that attracts ants and presents a unique adaptation to dispersal in the family (Caputo et al., 2004; Mayer and Ehrendorfer, 2013). Within *Knautia*, three sections corresponding to three basic karyotypes have been recognized (Ehrendorfer, 1962a, 1981). Most species of *Knautia* belong to the nearly exclusively perennial and most widespread sect. *Trichera*, which is characterised by a high incidence of polyploidy. Many-flowered capitula, relatively short corolla tubes and only slightly zygomorphic, mostly lilac flowers with generalized bee pollination, only ca. eight calyx bristles, and a relatively large genome with $x = 10$ (karyotype A) were suggested to be plesiomorphic traits (Ehrendorfer, 1962a, 1981). The exclusively annual and diploid sections *Tricheroides* (*K. byzantina*, *K. integrifolia*) and *Knautia* (*K. degenii*, *K. orientalis*) are restricted to the eastern Balkan Peninsula and northwestern Anatolia, only *K. integrifolia* extends to the central and western Mediterranean. With respect to their morphology and karyology these sections are not sharply separated from each other and from sect. *Trichera* from which they were suggested to differ in apomorphic tendencies (Ehrendorfer, 1962a, 1981). Among the annuals a gradual increase of apomorphies was postulated from *K. integrifolia* (sect. *Tricheroides*) to *K. orientalis* (sect. *Knautia*): reduction of the number of flowers per capitulum, elongation of corolla tubes, zygomorphic enlargement of outer corolla lobes, switch to a reddish-purple flower colour and butterfly pollination, increase of calyx bristles to 16 and more, and change from $x = 10$ to $x = 8$ (in *K. orientalis*, karyotype C; Ehrendorfer, 1962a). In addition, reduction of genome size in *K. integrifolia* (karyotype B) was observed (B. Frajman, unpubl. data).

In the last comprehensive treatment of sect. *Trichera*, Ehrendorfer (1962a, 1981) placed all taxa into eleven informal groups on the basis of morphology, distribution patterns and ploidy levels, and discussed their relationships. He recognized the two exclusively diploid groups of *K. montana* and *K. velutina*, the five diploid-tetraploid groups of *K. arvensis*, *K. dalmatica*, *K. dinarica*, *K. drymeia* and *K. longifolia* and the four exclusively polyploid (tetraploid and hexaploid) groups of *K. dipsacifolia*, *K. fleischmannii*, *K. sarajevensis* and *K. subcanescens/K. persicina*. In addition, several hybrids involving taxa with different ploidy levels and from different groups were mentioned.

According to Ehrendorfer (1962a, 1981) the *K. drymeia* group is clearly defined by monopodial growth and a central leaf rosette, a character not found in any other group. The taxa of this group are mostly forest species of which only *K. drymeia* comprises diploid cytotypes. The amphiadriatic *K. dinarica* group can be recognised by its mostly undivided leaves, soft indumentum with additional, mostly yellowish setae on basal parts. The taxa of this group (*K. csikii* and *K. dinarica*) mostly grow in upper montane to alpine meadows and tall herb communities. The *K. longifolia* group is characterised by undivided, mostly lanceolate leaves with entire margins and a tendency towards glabrousness. Besides *K. longifolia* distributed in the Alps and Carpathians, Ehrendorfer (1962a, 1981) included also *K. pancicii* from the Balkan Peninsula, *K. godetii* and *K. basaltica*, as well as some other polyploid species from the Western Alps, Massif Central and Pyrenees. Comparable to the members of the *K. dinarica* group they mostly grow in upper montane to alpine meadows and tall herb communities. The *K. montana* group includes *K. involucrata* and *K. montana* (incl. *K. tatarica*), yellow-flowering, partly tall herbs from Caucasus, Transcaucasus and Anatolia with ovate, undivided to lyrate leaves and a tendency towards monocarpic growth. The *K. velutina* group was characterised by not or only weakly divided leaves with one to three pairs of lobes and a dense lanuginose to tomentose indumentum; it has a strongly disjunct distribution from the southernmost Dinaric Alps (*K. albanica*) over the Velebit Mts. (*K. velebitica*) and the southern Alps (*K. carinthiaca*, *K. velutina*) to the southwestern Alps (*K. mollis*) and the Apennines (*K. calycina*). The *K. dalmatica* group, characterised by internodia shortened towards the stem base and regularly divided leaves with several linear to narrowly lanceolate lobes, includes *K. pectinata* as well as several polyploid taxa from the Dinaric Mountains, all growing in (sub)mediterranean grasslands. Related and growing in the same area but characterised by leathery and glossy leaves are the members of the *K. fleischmannii* group. The latter includes also *K. travnicensis*, which was recently shown to comprise also diploids (Frajman et al., unpubl.). Finally, Ehrendorfers *K. arvensis* group is morphologically very heterogeneous with mostly divided leaves and includes *K. arvensis* with a vast distribution area in western Eurasia, although its diploid members are geographically more restricted. Other diploid constituents are *K. ambigua* and *K. macedonica*, which are fairly similar to *K. arvensis*, as well as *K. purpurea* and the monocarpic *K. visianii*. All these species mostly grow in grasslands and ruderal places.

Molecular phylogenetic studies within the subfamily Dipsacoideae have so far focussed on generic relationships. *Knautia* was covered only by a single (Avino et al., 2009; Caputo et al., 2004) or by a handful of species (Carlson et al., 2009). This latter study has shown the genus to be monophyletic. Otherwise, *Knautia* has never been the subject of a more detailed molecular phylogenetic investigation. Available phylogenies based on nuclear ITS and plastid DNA markers have resolved the monotypic and annual Iberian *Pterocephalidium* as sister to *Knautia* (Avino et al., 2009; Caputo et al., 2004; Carlson et al., 2009), albeit with low support in the ITS phylogeny. This relationship is supported by the synapomorphic triporate pollen of the two genera (all other Dipsacoideae with the exception of *Pterothamnus* – see below – have tricolpate or tricolporate pollen) and has led Carlson et al. (2009) to suggest the inclusion of *Pterocephalidium* into Knautieae. Nevertheless, in a subsequent multidisciplinary (but not yet DNA-supported) study Mayer and Ehrendorfer (2013) have shown that *Pterocephalidium* is much closer to the newly described monotypic and shrubby African genus *Pterothamnus*, also with triporate pollen. Both genera differ strongly from *Knautia* by their in cross section round (not flattened) epicalyx with eight (instead of two) ribs and the lack of an elaiosome. Thus, both were separated from Knautieae and placed in a separate tribe, Pterocephalidieae. With respect to *Knautia*, Carlson et al. (2009) have demonstrated a sister relationship of perennial members of sect. *Trichera* (with $x = 10$) with the annual *K. orientalis* ($x = 8$) from sect. *Knautia*. Relationships among the few species included from sect. *Trichera* have remained largely unresolved due to shallow plastid DNA divergence and low support for ITS differentiation, which might well relate to the inclusion of polyploids with recombinant sequences.

The present study is an initial step towards unravelling the spatiotemporal diversification of *Knautia*. Our main objective is to provide the first comprehensive phylogenetic analysis of the genus by assessing relationships among diploid accessions, the raw material upon which polyploid evolution, particularly important in sect. *Trichera* (Ehrendorfer, 1962a), has been acting. The selection of these diploid samples was based on a flow-cytometric study of ca. 370 populations from almost all currently recognised taxa spanning from the Iberian Peninsula to the Caucasus (Frajman et al., unpubl.). We reconstructed phylogenies based on two unlinked gene regions, the nuclear ribosomal internal transcribed spacer (ITS) and the plastid *pet*N(*ycf*6)-*psb*M region. In addition, amplified fragment length polymorphisms (AFLPs) were obtained to increase resolution. In particular, our goals were (1) to test the monophyly of *Knautia* and its three sections *Trichera, Tricheroides* and *Knautia*, and (2) to consider the phylogenetic relationships

between perennial and annual members of the genus. Most important (3), we tested Ehrendorfer's (1962a) informal groups within the intricate sect. *Trichera* in order to elucidate the evolutionary relationships among its diploid species and to sketch a scenario of their spatiotemporal diversification.

2. Materials and methods

2.1. Species studied

We based the selection of samples on our comprehensive previous exploration of ploidy level variation in *Knautia*, focussing on sect. *Trichera* and including ca. 370 populations from ca. 50 species (Frajman et al., unpubl.). Based on this information, we selected diploid individuals from all 34 taxa where they were present, and aimed to include at least five individuals per taxon. All taxa including diploid cytotypes according to Flora Europaea (Ehrendorfer, 1976) were sampled; the exception is *K. dalmatica*, of which we encountered only tetraploid populations. As we were not interested in intra-population genetic diversity, we maximised the number of populations at the expense of the number of samples per population. Determinations were based on Flora Europaea (Ehrendorfer, 1976) and national floras (Devesa, 2007; Diklić, 1973; Ehrendorfer, 1982; Matthews, 1972). Herbarium vouchers were revised by F. Ehrendorfer, a taxonomic expert of the group.

2.2. Plant material

Leaf material of one to five individuals per population and one to 28 populations per species (i.e., roughly proportional to the size of the species' distribution areas; Fig. 1) was collected and immediately stored in silica gel. Exceptions are *K. byzantina* and population K473 of *K. orientalis*, of which herbarium material stored in the herbaria WU and W was used. We aimed at sampling morphologically and ecologically homogenous populations and avoided possibly hybridogenous individuals. Taxonomy follows Flora Europaea (Ehrendorfer, 1976) with the exception of the recently described *K. slovaca* (Štěpánek, 1983), the Iberian (Devesa, 2007) and Turkish taxa (Matthews, 1972), *K. collina* (Devesa, 2007) and *K. csikii* (Diklić, 1973).

Voucher specimens are either deposited at the Institute of Botany, University of Innsbruck, Austria (IB), the Faculty of Science,

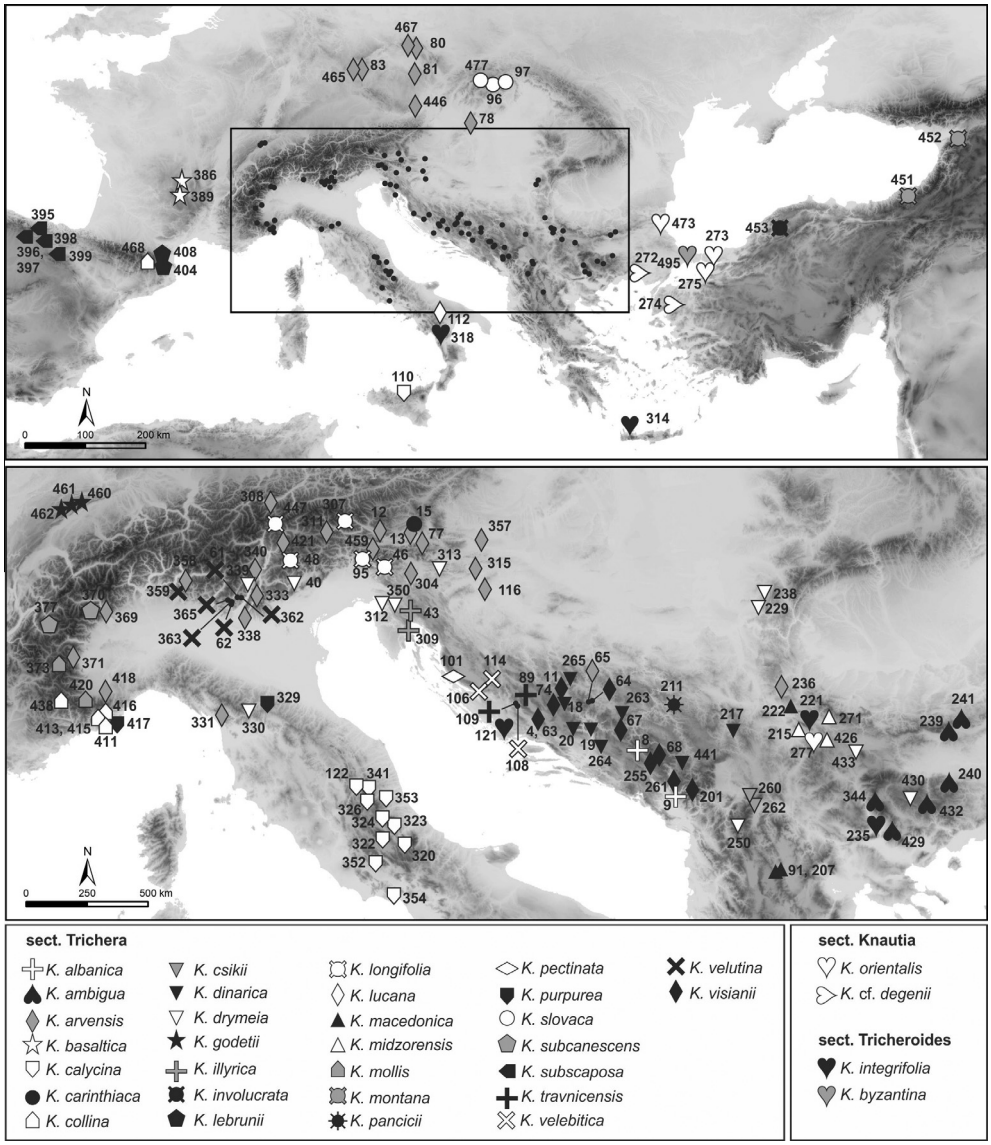

Fig. 1. Sampled diploid populations of 34 species of *Knautia*. Population identifiers correspond to Supplementary Table 1.

University of Zagreb, Croatia (ZA), the Faculty of Agriculture, University of Zagreb, Croatia (ZAGR), the Faculty of Biology, University of Belgrade, Serbia (BEOU) or the Natural History Museum Belgrade, Serbia (BEO). Voucher numbers and collecting details are given in the Supplementary Table S1; further information on the populations can be retrieved from the publicly accessible database of the BALKBIODIV project at <http://www.uibk.ac.at/botany/balkbiodiv/?Sampling_sites>.

2.3. Molecular methods

Total genomic DNA was extracted from similar amounts (ca. 10 mg) of dried tissue with the DNeasy 96 plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Exceptions are the herbarium samples of *K. byzantina* and *K. orientalis* that were extracted using a CTAB extraction protocol (Doyle and Doyle, 1987) with slight modifications (Schönswetter et al., 2002).

Five regions (*pet*N(*ycf*6)–*psb*M, *trn*L(UAG)-*ndh*F, *ndh*J-*trn*T, *trn*Q-*trn*K, *rps*16) of the plastid genome have been tested for variation. The plastid *pet*N(*ycf*6)-*psb*M region yielding the highest variability as well as the nuclear ribosomal ITS region were sequenced from one individual each of 148 populations plus nine and twelve outgroup accessions using the primers ycf6-F and psbM-R (Shaw et al., 2005) and 17SE and 26SE (Sun et al., 1994), respectively. The PCR products were obtained in reaction volumes of 16.5 µl including 6 µl REDTaq ReadyMix PCR reaction mix (Sigma–Aldrich, Steinheim, Germany), 0.7 µl of 1 mg/ml BSA (bovine serum albumin; Promega, Madison, WI), 0.4 µl 10 µM each of forward and reverse primers and ca. 25 ng DNA. The PCR conditions for ITS were as follows: 94 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C (with a decrease of 0.4 °C per cycle and a constant temperature of 48 °C from cycle 15 onwards) and 1 min at 72 °C; and a final elongation step of 10 min at 72 °C, while the PCR amplification for the plastid *pet*N(*ycf*6)-*psb*M region was carried out with initial denaturation at 94 °C for 2 min, 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 2 min extension at 72 °C, followed by 10 min final extension at 72 °C. PCR programs were run on Eppendorf 5331 thermocyclers (PE Applied Biosystems, Foster City, CA). PCR products were purified with *E. coli* Exonuclease I and SAP (Shrimp Alkaline Phosphatase; Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions. Cycle sequencing reactions were performed using 8 µl of purified template and 1 µl BigDye Terminator (PE Applied Biosystems), then cleaned with Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) and sequenced on an ABI 3770 DNA Analyzer (PE Applied Biosystems).

AFLP fingerprinting was performed for 136 populations (excluding *K. byzantina*, *K.* cf. *degenii* and *K. orientalis*, from which either only herbarium material was available or which were received too late to be included in the AFLP data set) with one to five individuals per population, in total including 189 individuals. The AFLP procedure followed Vos et al. (1995) with the modifications described in Schönswetter et al. (2009). As additional modification, 0.25 U of polymerase were used in the preselective and selective amplifications (0.4 U for the NED-labelled primer combination). Initially, selective primers were screened using 12 primer combinations. The three final primer combinations for the selective PCR (fluorescent dye in brackets) were *Eco*RI (6-FAM)-ACA/*Mse*I-CTG, *Eco*RI (VIC)-ACG/*Mse*I-CTA, and *Eco*RI (NED)-ACC/*Mse*I-CTC. Purification and visualisation of PCR products were done as described in Rebernig et al. (2010). Nineteen samples were used as replicates between PCR plates to test reproducibility.

Electropherograms of AFLP fingerprints were analysed with Peak Scanner version 1.0 (Applied Biosystems) using default peak detection parameters except a light peak smoothing. The fluorescent threshold was set to 50 relative fluorescence units. Automated binning and scoring was performed using RawGeno version 2.0 (Arrigo et al., 2009), a package for the software R (R Development Core Team, 2012), with the following settings: scoring range = 50–500 bp, minimum intensity = 100 rfu, minimum bin width = 1 bp, and maximum bin width = 1.5 bp. Fragments with a reproducibility lower than 80% based on sample-replicate comparisons were eliminated. The error rate (Bonin et al., 2004) was calculated as the ratio of mismatches (scoring of 0 vs. 1) over phenotypic comparisons in AFLP profiles of replicated individuals. Non-reproducible fragments and fragments present in only one individual were removed from the dataset.

2.4. Data analysis

2.4.1. Sequence data

Sequences were edited using Geneious Pro 5.3.6 (Drummond et al., 2011). Base polymorphisms were coded using the NC-IUPAC ambiguity codes; the polymorphisms were scored when the weaker signal reached at least one third of the height of the stronger signal in both strands. Sequences were then manually aligned using QuickAlign (Müller and Müller, 2003) without problems. All sequences were deposited in GenBank (Supplementary Table S1). Phylogenetic relationships of both the ITS and the plastid data sets were inferred using maximum parsimony as well as Bayesian analyses. The ITS dataset included 151 new sequences and nine GenBank sequences, while the plastid dataset included 157 newly generated sequences. Maximum parsimony (MP) as well as MP bootstrap analyses of both data sets were performed using PAUP 4.0b10 (Swofford, 2002). The most parsimonious trees were searched heuristically with 1000 replicates of random sequence addition, TBR swapping, and MulTrees on. The swapping was performed on a maximum of 1000 trees (nchuck = 1000). All characters were equally weighted and unordered. The data set was bootstrapped using full heuristics, 1000 replicates, TBR branch swapping, MulTrees option off, and random addition sequence with five replicates. *Bassecoia hookeri* (=*Pterocephalus hookeri*) was used to root the trees and some additional outgroup taxa from different genera closely related to *Knautia* (Carlson et al., 2009) were also included in the analyses (Supplementary Table S1).

Bayesian analyses were performed employing MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), using the parallel version (Altekar et al., 2004) at the computer cluster Bioportal at the University of Oslo (http://www.bioportal.uio.no/) applying the substitution models proposed by the Akaike information criterion implemented in MrAIC.pl 1.4 (Nylander, 2004; Table 1). Values for all parameters, such as the shape of the gamma distribution, were estimated during the analyses. The settings for the Metropolis-coupled Markov chain Monte Carlo (MC^3) process included four runs with four chains each (three heated ones using the default heating scheme), run simultaneously for 10,000,000 generations each, sampling trees every 1000th generation using default priors. The PP of the phylogeny and its branches was determined from the combined set of trees, discarding the first 1001 trees of each run as burn-in.

We constructed an ITS NeighbourNet network (Bryant and Moulton, 2004) of sect. *Trichera* using SplitsTree 4.12 (Huson, 1998; Huson and Bryant, 2006) to display possible conflicts in the data. We applied the Uncorrected_P method to compute the proportion of positions at which two sequences differ. Ambiguous base codes were treated as missing states. Plastid data were also analysed using statistical parsimony as implemented in TCS (Clement et al., 2000) with the connection limit set to 95%. Since gaps were treated as fifth character state, indels longer than 1 bp were re-coded as single characters by reducing them to single base pair columns.

We reconstructed ancestral states for the character "life form" (annual, biennial, perennial; assignment of species based on

Table 1
Matrix and phylogenetic analysis statistics for ITS and the plastid marker *pet*N(*ycf*6)-*psb*M as well as substitution models proposed by MrAIC and used in the Bayesian analyses.

	***pet*N(*ycf*6)-*psb*M**	**ITS**
Number of terminals	157	161
Number of included characters	1398	953
Number/percentage of parsimony informative characters (within the ingroup)	95 (50)/6.8% (3.6%)	182 (106)/19.1% (11.1%)
Number of MP trees	260	386,000
Length of MP trees	210	510
Consistency index (CI; excluding uninformative characters)	0.895 (0.827)	0.663 (0.594)
Retention index (RI)	0.960	0.908
Substitution model	GTR + Γ	GTR + Γ

Devesa, 2007; Ehrendorfer, 1976; Hong et al., 2011 and Matthews, 1972) using Mesquite (Maddison and Maddison, 2010), with the "Trace Character Over Trees" module applying the parsimony reconstruction method over all trees derived from the MrBayes analyses of the pruned concatenated plastid and ITS data sets (analysed in the same way as the complete data sets; see above), discarding the first 1001 trees of each run as burn-in. Combinability of the pruned plastid and ITS data sets was assessed in a parsimony framework using the incongruence length difference (ILD) test implemented in PAUP 4.0b10 (Swofford, 2002) employing 1000 partition replicates, each with 10 random sequence addition replicates saving no more than 500 trees per replicate and TBR branch swapping. A significance threshold of 0.01 was applied.

2.4.2. AFLP data

A Neighbour-joining (NJ) analysis based on a matrix of Nei-Li distances (Nei and Li, 1979) and rooted with *K. integrifolia* from section *Tricheroides* was conducted and bootstrapped (1000 pseudo-replicates) with TREECON 1.3b (Van de Peer and De Wachter, 1997). Using SplitsTree 4.12 (Huson and Bryant, 2006), a Neighbor-Net diagram was produced from a matrix of uncorrected P distances; *K. integrifolia* with strongly divergent AFLP profiles was not included. Splits with a weight <0.001 were excluded to aid legibility.

3. Results

The number of terminals, included characters, parsimony informative characters, percentage of parsimony informative characters, number and lengths of MP trees, consistency and retention indices for both DNA regions, as well as the model of evolution proposed by MrAIC and used in MrBayes analyses are presented in Table 1.

3.1. Plastid sequence data

The *pet*N(*ycf*6)-*psb*M sequences were 1230–1273 bp long and the alignment was 1398 bp long (Table 1). Monophyly of *Knautia* is strongly supported (bootstrap support, BS 96, PP 1.00, Fig. 2, Supplementary Fig. S1) and a sister relationship with *Pterocephalidium* is resolved (BS 69, PP 0.92, Fig. 2, Supplementary Fig. S1). Within *Knautia* three highly supported clades were resolved, corresponding to the three sections *Knautia* (BS 100, PP 1.00), *Tricheroides* (but including one accession of *K.* cf. *degenii*; BS 99, PP 1.00) and *Trichera* (BS 99, PP 1.00), the latter two forming a sister group (BS 95, PP 1.00). Within sect. *Trichera* several clades with poorly resolved and insufficiently supported relationships were unravelled (Supplementary Fig. S1).

The parsimony network of plastid DNA haplotypes sampled in sect. *Trichera* (Fig. 3C, Supplementary Fig. S3) exhibited a simple

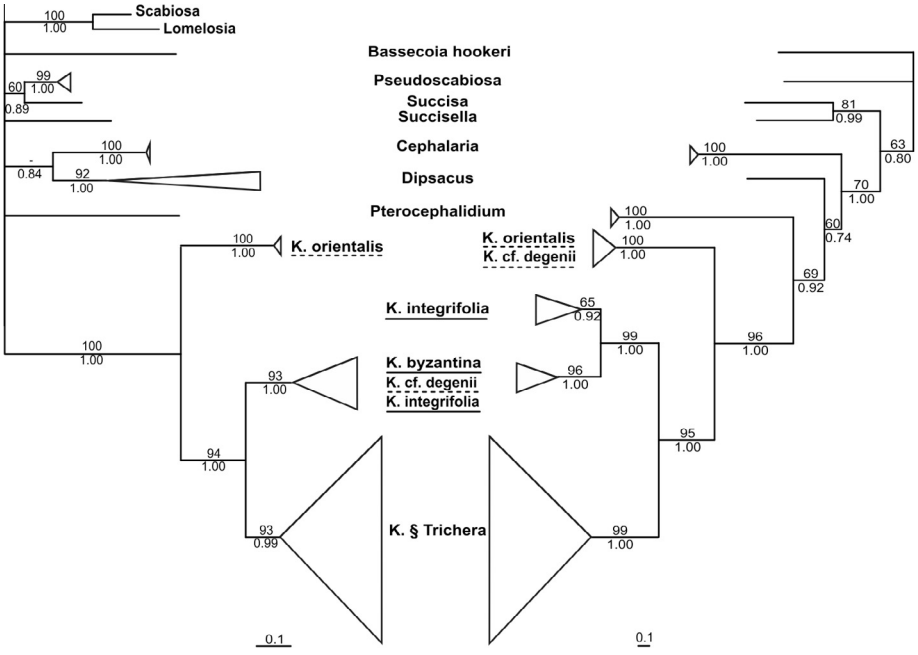

Fig. 2. Reconstructions of phylogenetic relationships in Dipsacoideae focussing on *Knautia*. Left, simplified phylogram based on Internal Transcribed Spacer (ITS) sequences presented in detail in Supplementary Fig. S2. Right, simplified phylogram based on the plastid *pet*N(*ycf*6)-*psb*M region presented in detail in Supplementary Fig. S1. Values above and below branches are parsimony bootstrap values >50 and posterior probabilities derived from Bayesian analysis >0.80, respectively. Branches obtaining lower support in both analyses were collapsed. Continuous and dotted underlinings mark members of *K.* sect. *Tricheroides* and *K.* sect. *Knautia*, respectively.

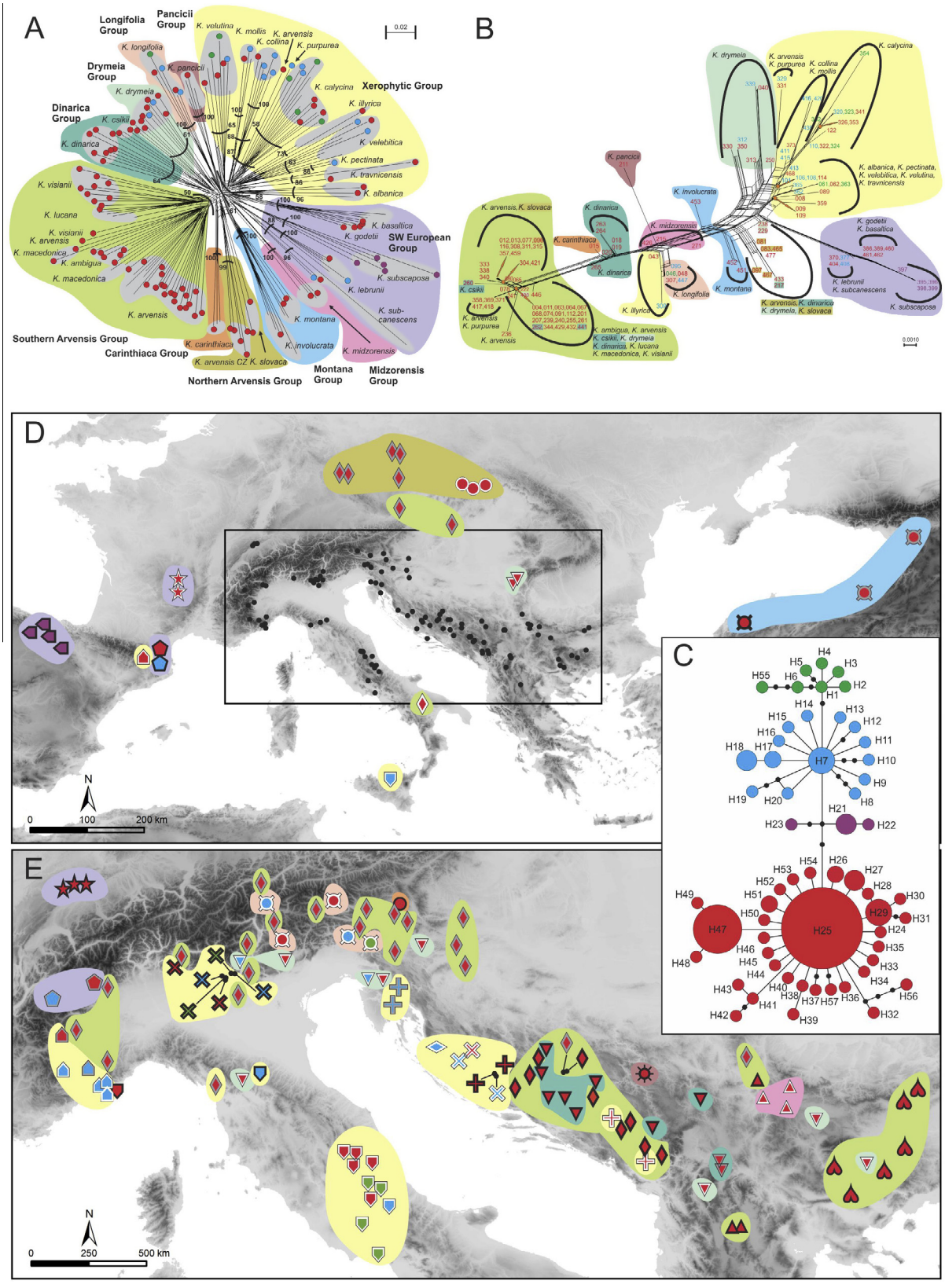

Fig. 3. Structuring and geographical distribution of AFLP, plastid *pet*N(*ycf*6)-*psb*M and nuclear ribosomal ITS variation in diploid populations of 30 species of *Knautia* sect. *Trichera*. (A–B) NeighbourNet diagrams based on uncorrected P distances. The AFLP NeighbourNet (A) is complemented with bootstrap values >50% (given for major groups of individuals only) derived from the neighbour-joining analysis shown in Supplementary Fig. S4. Colouring of dots (A) and population IDs (B) at the tips of the branches corresponds to the groups of plastid DNA haplotypes shown in (C). In (A), major AFLP groups are shaded in colour; the shading in (B) follows these AFLP groups. (C) Statistical parsimony network of the 57 plastid haplotypes encountered (numbering corresponds to Supplementary Table S1; the size of the circles is proportional to the square-root transformed frequency of the respective haplotype; haplotypes not sampled are shown as small black dots). (D–E) geographical distribution of plastid haplotypes. Symbols for the species are as in Fig. 1; their colour filling corresponds to the haplotype groups shown in C.

structure. In total, 57 haplotypes were retrieved (from here on, the term haplotype is strictly restricted to plastid sequences). The most frequent haplotype H25 was found in 34.5% of the samples. It connects to 29 closely related haplotypes differing in only one or two steps, only H31 and H42 are separated by three steps (Red Haplotype Group in Fig. 3). One of the satellites of H25 is H47, the second-most frequent haplotype (11.8%). The most distinct haplotype (H56) was found in a sample of *K. drymeia* from Romania and was separated by four mutational steps. Three haplotypes (H21–H23, Violet Haplotype Group in Fig. 3) exclusively found in Spanish *K. subscaposa* are connected to a not sampled haplotype, to which also H7 is connected. The latter is surrounded by 13 haplotypes separated by maximally three steps (Blue Haplotype Group in Fig. 3). Finally, H1, giving rise to six haplotypes separated by one to five steps, is connected to H7 by two steps (Green Haplotype Group in Fig. 3).

The geographic distribution of the haplotype groups is shown in Fig. 3. The Red Haplotype Group is distributed throughout most of the sampling area, ranging from the Pyrenees to the Caucasus. This was the only haplotype group sampled north of the Alps as well as in the eastern parts of the Balkan Peninsula east of Mt. Dinara and eastwards to the Caucasus. The second-most widely distributed haplotype group (Blue Haplotype Group) ranges from the eastern Pyrenees over the Alps to the Apennines and Sicily, as well as to the westernmost Balkan Peninsula. The Green Haplotype Group is restricted to *K. calycina* from the Apennines and three accessions of *K. longifolia* and *K. velutina* from the southern Alps. The Violet Haplotype Group is the most restricted, present only in populations of *K. subscaposa* from the northern Iberian Peninsula (populations K395–K399). The centre of haplotype diversity within sect. *Trichera* extends from the southern Alps to the central Apennines. Plotting the haplotypes retrieved from each species onto the parsimony network (Supplementary Fig. S3) illustrates that all frequent haplotypes are shared across taxa. For instance, the most frequent haplotype H25 was found in 21 out of 30 species. It is also evident that individuals of some species, such as *K. calycina*, *K. drymeia*, *K. longifolia* and *K. velutina* harbour unrelated haplotypes from different haplotype groups.

3.2. ITS sequence data

Raw ITS sequences were 845–868 bp long and the alignment was 953 bp long (Table 1). Eighty out of 137 accessions from sect. *Trichera* contained polymorphic positions; half of them were polymorphic only at one site, whereas 11 accessions contained more than three polymorphic sites (Supplementary Table S1). *Knautia* was resolved as monophyletic and highly supported (BS 100, PP 1.00, Fig. 2, Supplementary Fig. S2), but the relationships with the outgroup genera remain unclear. Within the genus three well supported clades (BS 93–100, PP 0.99–1.00) roughly corresponding to the three sections were inferred. Section *Knautia* was highly supported (BS 94, PP 1.00) as sister to the remaining two clades. Within sect. *Trichera* several clades were poorly resolved and insufficiently supported. Both accessions of *K.* cf. *degenii* were positioned within *K. integrifolia*.

The NeigbourNet network of ITS ribotypes (Fig. 3B; Supplementary Fig. S5) revealed a deep split between two major groups and some taxa positioned along the split (*K. carinthiaca*, *K. dinarica*, *K. illyrica*, *K. longifolia*, *K. midzorensis* and *K. pancicii*). Whereas the first group including most taxa of the *K. arvensis* alliance (sensu Ehrendorfer, 1962a), *K. csikii* and a few samples of *K. drymeia* and *K. dinarica* is fairly homogenous and exhibits low genetic diversification, the second group comprises all other taxa (together with a few samples of *K. arvensis* from the Northern Arvensis Group (see below), *K. dinarica* and *K. drymeia* and is genetically highly diverse, with several strongly weighted splits.

3.3. Combined sequence data and ancestral state reconstruction of the life form

Even if there were conflicts detected between the pruned datasets of both DNA regions by the ILD test ($P = 0.001$), we conducted the ancestral life form reconstruction on the trees obtained by the Bayesian analysis of the concatenated dataset. The incongruences were likely caused by conflicts in the topology of the outgroup taxa (see Fig. 2) or within the main ingroup lineages and are therefore not important when reconstructing the ancestral character states of the main ingroup lineages, whose position is highly congruent in both phylogenetic trees. The common ancestor of the genus *Knautia*, as well as the common ancestor giving rise to sections *Trichera* and *Tricheroides* was inferred as annual in 88% of the trees (in 12% the reconstruction was equivocal; Supplementary Fig. S6). The common ancestor of both sects. *Knautia* and *Tricheroides* was inferred as annual, and that of sect. *Trichera* as perennial.

The analysis of the combined dataset (Supplementary Fig. S6) inferred *Knautia* as monophyletic with strong support (PP 1.00) and suggested the same relationships among the three sections as the analyses of separate datasets (Fig. 2). *Pterocephalidium* was inferred as sister to *Knautia*, albeit with lower support (PP 0.89). Within sect. *Trichera* some groups of taxa received low to moderate support (PP 0.93–0.97). The composition of highly supported clades reflected either highly supported ITS clades (e.g., the taxa of the SW European and Southern Arvensis groups; Supplementary Fig. S2), or plastid clades (e.g., the grouping of *K. illyrica* and *K. longifolia;* Supplementary Fig. S1).

3.4. AFLP data

Five individuals failed to produce reliable AFLP profiles and were excluded, resulting in a final dataset including 184 individuals. A total of 897 AFLP fragments were scored; 80 bands were found in only one individual and one fragment was monomorphic. These latter fragments were excluded from further analyses. The average replicate error rate (according to Bonin et al., 2004) was 2.04%. The NeighbourNet analysis of sect. *Trichera* was based on a matrix with 179 individuals and 808 AFLP fragments.

The NJ analysis of AFLP data (Supplementary Fig. S4) supports the divergence between *K. integrifolia* from sect. *Tricheroides* and the species of sect. *Trichera* (BS 100). *Knautia pancicii* (BS 100; Pancicii Group) appears as sister to the remaining species with low support (BS 57). Species forming well-supported (BS $\geqslant$95) branches include *K. albanica*, *K. carinthiaca*, *K. collina*, *K. involucrata*, *K. lebrunii*, *K. longifolia*, *K. midzorensis*, *K. mollis*, *K. subcanescens* and *K. subscaposa*. *Knautia carinthiaca*, *K. longifolia* and *K. midzorensis* constitute separate groups (named Carinthiaca Group, Longifolia Group and Midzorensis Group in the following). *Knautia basaltica* and *K. godetii* are intermingled, but together form a maximally supported branch. The same is true for *K. arvensis* from north of the Alps. The backbone of the tree is unresolved, and the support values are low. Accessions of *K. arvensis* (excluding those from north of the Alps) cluster together with those of *K. ambigua* and *K. macedonica*. In addition, *K. visianii* and the nested *K. lucana* fall into this weakly supported (BS 50) group (Southern Arvensis Group). Accessions of *K. drymeia* are separated into two geographically distant groups spanning the Alps (BS 75) and the Carpathians (BS 71) and adjacent areas. Both branches cluster with low support (BS 61; DrymeiaGroup). The next, though unsupported, relatives are *K. dinarica* and the nested *K. csikii* forming the Dinarica Group (BS 64). Accessions of *K. arvensis* from north of the Alps cluster with *K. slovaca*, albeit without support (Northern Arvensis Group). *Knautia involucrata* is nested within *K. montana* with maximal support (BS 100; Montana Group). Several species distributed from the Iberian Peninsula over the Massif Central to the Jura Mountains form

an unsupported group (SW European Group), which falls into three well-supported sub-branches. The first (BS 88) combines *K. lebrunii* and *K. subcanescens*, the second *K. subscaposa* (BS 100) and the third *K. basaltica* and *K. godetii* (BS 100). Finally, several drought-adapted taxa form a weakly supported (BS 58) cluster (Xerophytic Group) comprising (1) an unsupported group formed by *K. albanica*, *K. illyrica*, *K. pectinata*, *K. travnicensis* and *K. velebitica*, (2) *K. velutina* (BS 65), and (3) a relatively better supported (BS 87) cluster containing *K. collina* and *K. mollis* (together BS 88), and a weakly supported (BS 57) group from Italy dominated by *K. calycina* and single accessions of *K. arvensis* and *K. purpurea*.

The NeighbourNet analysis of the AFLP data (Fig. 3A; Supplementary Fig. S5) yielded congruent results. In addition, the distinctness of the Southern Arvensis Group is underlined by a set of comparatively long splits.

4. Discussion

4.1. Position of Knautia and its sectional classification

Knautia is clearly resolved as monophyletic (Fig. 2). In agreement with previous studies (Caputo et al., 2004; Carlson et al., 2009), the genetic divergence of the genus justifies recognition as the only member of tribe *Knautieae*. It is distant sister to the monotypic annual genus *Pterocephalidium* from the Iberian Peninsula (albeit with low support: Fig. 2, Supplementary Figs. S1, S2 and S6), the latter forming the tribe *Pterocephalidieae* (probably together with the African genus *Pterothamnus*; Mayer and Ehrendorfer, 2013). The traditional recognition of three sections within *Knautia* is largely supported (Fig. 2, Supplementary Figs. S1 and S2). The main bulk of species forms the monophyletic sect. *Trichera*, which comprises mainly perennial, but also a few monocarpic, biennial species (*K. montana*, *K. visianii*). *Knautia byzantina* and *K. integrifolia* constitute a monophyletic sect. *Tricheroides*, and all populations of *K. orientalis* from sect. *Knautia* form a maximally supported clade. The position of two accessions (K272, K274) ambiguously determined as *K.* cf. *degenii* (the second species of section *Knautia*, morphologically intermediate between *K. integrifolia* and *K. orientalis*) is uncertain due to incongruences between nuclear and plastid markers (Fig. 2). According to ITS both accessions are linked with *K. integrifolia* from sect. *Tricheroides* (Supplementary Fig. S2), whereas in the plastid phylogeny K272 is positioned within sect. *Knautia*, K274 within sect. *Tricheroides* (Supplementary Fig. S1). This conflicting phylogenetic position may indicate intersectional hybridisation; lineage sorting is unlikely to be responsible for this pattern as the branches leading to both clades are relatively long, leaving enough time to achieve reciprocal monophyly (Frajman et al., 2009; Hudson and Coyne, 2002).

4.2. Evolution of perennials from annuals or vice versa?

Variation in life history strategies, such as the dichotomy between monocarpy and polycarpy has received considerable attention (Stearns, 1992; Young and Augspurger, 1991). A widely held opinion states that annuals are derived from perennial ancestors and that shifts between these two strategies are unidirectional (e.g., Stebbins, 1957). A well-documented case for such an origin of an annual species from perennial ancestors is the diploid *Arabidopsis thaliana* with $x = 5$, derived from perennial ancestors (comparable to *A. lyrata* or *A. halleri* with $x = 8$) by several steps of major genome reorganisation (Mitchell-Olds, 2001; Yogeeswaran et al., 2005). Congruently, many other studies phylogenetically resolved the evolution of annuals from perennials (e.g. in *Euphorbia*: Frajman and Schönswetter, 2011; *Veronica*: Albach et al., 2004). On the other hand, several recent phylogenetic studies have inferred the evolution of perennials from annual ancestors, e.g. in *Androsace* (Primulaceae; Schneeweiss et al., 2004), *Castilleja* (Orobanchaceae; Tank and Olmstead, 2008), *Delphinium* (Ranunculaceae; Jabbour and Renner, 2012), *Lupinus* (Fabaceae; Drummond et al., 2012) or in the legume tribe Fabeae (Schaefer et al., 2012).

Our phylogenies (Fig. 2, Supplementary Figs. S1 and S2) resolve the annual *K. orientalis* on a long branch as sister to a clade formed by the annual sect. *Tricheroides* and the perennial sect. *Trichera*. The reconstruction of life histories along the Bayesian trees inferred from the concatenated ITS and plastid sequences (Supplementary Fig. S6) thus suggests the evolution of perenniality in sect. *Trichera* from an annual ancestor. One of the factors potentially distorting phylogenetic relationships is long-branch-attraction (Bergsten, 2005). Branches leading to annual species are often longer as compared to those of perennials, reflecting shorter generation times and thus faster substitution rates in annuals (e.g., Frajman and Schönswetter, 2011; Smith and Donoghue, 2008; Tank and Olmstead, 2008). However, as perennial *Knautia* regularly flower already from the second year onwards (P. Schönswetter, personal observations from ca. 20 species cultivated in the Botanical Garden of the University of Innsbruck), it appears unlikely that the early diverging phylogenetic position of the annual species within *Knautia* is an artefact caused by strongly divergent generation times. Phylogenetic analyses without the annual *Pterocephalidium*, whose long branch might have attracted the branches of annual *Knautia* lineages, inferred the same topology among the three sections of *Knautia* (results not shown). Moreover, the same topology was obtained with both parsimony and Bayesian analyses, as well as with plastid sequences and ITS, rendering a topological artefact due to long-branch attraction unlikely (Bergsten, 2005).

The inference that annuality is plesiomorphic in *Knautia* is contrary to Ehrendorfer's (1962a, 1981) hypothesis that *K. orientalis* (sect. *Knautia*) evolved out of perennial members of sect. *Trichera*, to which it was suggested to be morphologically linked via the monocarpic *K. visianii* (sect. *Trichera*) and members of sect. *Tricheroides* (*K. integrifolia*) and sect. *Knautia* (*K. degenii*). Whereas origin of *K. orientalis* from extant members of sect. *Trichera* is clearly rejected by our data which rather suggest tight relationships among all species of sect. *Trichera* (see below) and strong divergence of sects. *Tricheroides* and *Knautia* (Fig. 2, Supplementary Figs. S1 and S2), we urge for cautious interpretation of our reconstruction of ancestral life form (Supplementary Fig. S6). The annual sects. *Knautia* and *Tricheroides* do not only comprise the morphologically and karyologically most divergent species, but are also genetically most divergent. It is thus not possible to exclude that perenniality is a plesiomorphic trait in *Knautia* and that evolution towards annual life strategies occurred twice along the long branches leading to sections *Tricheroides* and *Knautia*, respectively. Our reconstruction is influenced by the annual life form of *Pterocephalidium*, sister to *Knautia*, but if the perennial *Pterothamnus* is indeed most closely related to *Pterocephalidium*, the reconstruction of the life form of the ancestor of *Knautia* would likely be ambiguous. Similarly as in *K. orientalis*, in the genera *Cephalaria*, *Lomelosia* and *Pterocephalus* all perennial taxa studied by Verlaque (1985–1986) have $x = 9$ in contrast to the diploid annual *C. syriaca* ($x = 5$), *L. brachiata* ($x = 7$) and *P. brevis* ($x = 8$). Whereas Verlaque (1985–1986) postulates transitions from perennial to annual taxa, Carlson et al. (2009) also consider reverse changes. Finally, it is obviously necessary to obtain additional accessions of the annual *Knautia* species from the southeastern Balkans and adjacent Anatolia and the related western Mediterranean annual *Pterocephalidium diandrum* (Mayer and Ehrendorfer, 2013) as well as from not yet sequenced presumed relatives such as the African treelet *Pterothamnus* (known only from its type collection). This would facilitate more solid reconstructions of phylogenetic relationships as well as of the evolution of life history strategies and morphological traits.

4.3. Genetic structure among diploid members of Knautia sect. Trichera

The species-rich sect. *Trichera* was strongly supported as monophyletic in both nuclear and plastid phylogenies (Fig. 2, Supplementary Figs. S1 and S2). Both sequence markers and AFLPs, however, largely failed to resolve the internal phylogenetic relationships. The star-like structure exhibited in the ITS and AFLP data (Fig. 3), lack of resolution and of support in the phylogenetic reconstructions (Supplementary Figs. S1 and S2), as well as wide geographic ranges of both, single haplotypes and of groups of haplotypes and ribotypes (Fig. 3: haplotype H25, Red Haplotype Group; Southern Arvensis Group, Xerophytic Group), are indicative for (a) a young age of *Knautia* sect. *Trichera*, (b) rapid radiation and recent range expansion and/or (c) recurrent gene flow across species boundaries. According to divergence time estimates for Dipsacoideae (Carlson et al., 2012) the timing of the diversification of sect. *Trichera* can tentatively be placed in the Pleistocene, but dating analyses with wider outgroup sampling are needed to confirm this.

The weak geographic structure seen in the distribution of plastid haplotypes is also reflected by extensive haplotype sharing across species and the occurrence of unrelated haplotypes within several species (Supplementary Fig. S3). This is especially evident in *K. calycina*, *K. longifolia* and *K. velutina*, which harbour haplotypes of three groups in spite of nuclear homogeneity as indicated by both ITS and AFLPs (Fig. 3). Such a pattern may be explained by frequent hybridisation/introgression and/or the presence of shared ancestral polymorphisms (Schaal and Olsen, 2000). Both processes are difficult to decipher (Yu et al., 2011) and the latter can certainly not be excluded in a species group with low molecular differentiation, an obviously recent origin and relatively large effective population sizes (Fuertes Aguilar and Nieto Feliner, 2002; Gutiérrez Larena et al., 2002; Willyard et al., 2009).

Most ITS ribotypes fall into two large groups with only a few accessions from, amongst others, *K. dinarica*, *K. involucrata*, *K. longifolia*, *K. midzorensis*, *K. montana* and *K. pancicii* being positioned between them (Fig. 3B). Similar to the haplotype groups, both main ribotype groups are distributed over large geographic areas (Fig. 3). One group that largely corresponds to the Southern Arvensis Group based on AFLPs (Fig. 3A) comprises *K. arvensis* and morphologically allied taxa (plus single samples of *K. csikii*, *K. dinarica* and *K. slovaca*) and is also genetically fairly uniform (Fig. 3B, left). Almost all accessions of this group come from the Alps and the Balkan Peninsula (Fig. 3) and they all possess haplotypes of the Red Haplotype Group (Fig. 3). The second ribotype group is genetically and taxonomically more diverse (Fig. 3B, right) and is distributed across the entire area of sect. *Trichera* (Fig. 3). Accessions pertaining to this group possess haplotypes from all four haplotype groups; in the AFLP NeighbourNet (Fig. 3A) they are positioned in different groups, partly corresponding to those in the ITS NeighbourNet (Fig. 3B).

4.4. Systematics of Knautia sect. Trichera: a case for informal classification only?

Due to difficult species delimitation, common hybridisation and reticulate evolution often involving polyploidisation only two attempts of an intrasectional classification of sect. *Trichera* exist. The first was provided by Szabó (1911), who recognised several subsections and series in a strictly formal system. On the basis of karyological investigations and morphological observations Ehrendorfer (1962a) rejected the classification of Szabó (1911) as artificial and impracticable. He grouped the taxa into informal groups, taking the morphological differentiation of the diploid taxa as a basis for a system into which he inserted the polyploids (see Section 1). Consequently, most of his groups extend across cytotypes. Even if not taxonomically formalised, this approach provides explicit hypotheses on phylogenetic relationships.

For instance, Ehrendorfer (1962a) proposed a morphologically clearly distinct *K. drymeia* group including diploid and tetraploid *K. drymeia* and the little-known *K. gussonei* Szabó, both with monopodial rhizomes, undivided ovate leaves with strong dentation, hirsute stems usually with several reddish-violet flower heads and a preference of planar to subalpine forest habitats or tall herb communities. *Knautia gussonei* is only known from the type collection and plants collected by us close to the locus classicus in the Central Apennines were morphologically and genetically indistinguishable from tetraploid *K. drymeia* (Frajman et al., unpublished AFLP data). The AFLP data (Fig. 3A, Supplementary Fig. S4) support such a **Drymeia Group** (a comparison of the species goups proposed by us with those of Ehrendorfer (1962a, 1976, 1981) is provided in Table 2) with moderate support (BS 61), comprising all samples of the species. Nevertheless, a phylogenetically basal (i.e., early diverging) position of *K. drymeia* in sect. *Trichera* as suggested by Ehrendorfer (1962a) is not supported. The AFLP substructure of diploid *K. drymeia* (Supplementary Fig. S4) follows its disjunct distribution, with one group ranging from the northern Apennine Peninsula over the southern margin of the Alps to the Pannonian area (BS 75), and the second one being restricted to the southeastern Carpathians and the central Balkan Peninsula (BS 71). The corresponding ribotypes are, however, not monophyletic (Fig. 3B). In the phylogenetic analysis of the ITS data (Supplementary Fig. S2) the western populations grouped in two moderately supported clades which shared several splits in the NeighbourNet (Fig. 3B), supporting their common origin. Both western groups possessed red and blue haplotypes (Fig. 3). In contrast, the eastern populations possessed haplotypes from the Red Haplotype Group only (Fig. 3), but had more diverse ribotypes (Fig. 3B, Supplementary Fig. S2) exhibiting connections with western *K. drymeia* as well as *K. arvensis* and *K. dinarica*, two species known to hybridise with *K. drymeia* (Ehrendorfer, 1962a; Szabó, 1911). Several polymorphisms in ITS sequences of two accessions from Romania (K229, K238) may indicate recent hybrid origin, assuming that concerted evolution following hybridisation usually converges ITS copies towards one of the parents (Álvarez and Wendel, 2003).

Similarly conflicting patterns can be observed within the **Dinarica Group**. It is characterised by a sympodial rhizome, broadly lanceolate to ovate and toothed (but rarely lobed) leaves, prefers open montane grassland habitats, and includes *K. csikii* and *K. dinarica* from the western Balkan Peninsula (Ehrendorfer, 1962a). Ehrendorfer's hypothesis that this group has originated from the *K. drymeia* group is neither rejected nor supported by our data, as they are unsupported sister groups according to AFLP (Fig. 3A, Supplementary Fig. S4) but fairly distant in the ITS NeighbourNet (Fig. 3B). Monophyly of the Dinarica Group received moderate support (BS 64) in the AFLP analysis (Supplementary Fig. S4), but no support was inferred from ITS data where some samples displayed connections to both the northern (K217) and the southern (K441; also *K. csikii* K260 and K262) *K. arvensis* alliances (Fig. 3B). Again, hybridisation and gene flow from *K. arvensis* might be responsible for the observed pattern, even if most of the ITS sequences of *K. dinarica* were not polymorphic. Occasional individuals with divided leaves (as characteristic for *K. arvensis*) found within populations of *K. dinarica* (B. Frajman and P. Schönswetter, pers. field obs.), may indicate such introgression.

Ehrendorfer's (1962a) *Knautia longifolia* group comprising *K. longifolia*, *K. midzorensis*, *K. pancicii* and some western European species (see below), corresponds widely with Szabó's (1911) subsect. *Silvaticae* and is characterized by sympodial rhizomes, undivided lanceolate leaves with much reduced or mostly lacking dentation, glabrous or soft pubescent stems, often with only one

Table 2
Comparison of the species groups within *Knautia* sect. *Trichera* proposed by us with those of Ehrendorfer (1962a, 1981).

AFLP groups and constituent species	Ehrendorfers (1962a, 1981) groups	Habitat preference of individual species	Distribution type of AFLP groups and distribution of individual species	Longevity: mono- or polycarpic	Morphological characteristics of AFLP groups
Southern Arvensis Group			Widespread	m-p	Leaves usually divided
K. ambigua	*K. arvensis* group	Grasslands and ruderal places	SE Balkan Peninsula	p	
K. arvensis	*K. arvensis* group	Grasslands and ruderal places	Most of Europe except for the extreme south	p	
K. lucana	*K. arvensis* group	Forests	S Italy	m	
K. macedonica	*K. arvensis* group	Grasslands and ruderal places	Central Balkan Peninsula	p	
K. visianii	*K. arvensis* group	Grasslands and ruderal places	Central and W Balkan Peninsula	m	
Northern Arvensis Group			Widespread	p	Leaves usually divided
K. arvensis	*K. arvensis* group	Grasslands and ruderal places	Most of Europe except for the extreme south	p	
K. slovaca	–	Natural grasslands on limestone	Central eastern Slovakia	p	
Carinthiaca Group			Narrow endemic	p	Leaves divided with long terminal segment; soft indumentum; flowers pale pink
K. carinthiaca	*K. velutina* group	Natural grasslands and rock crevices, limestone	Austria (E Central Alps)	p	
Dinarica Group			Regional, continuous	p	Mostly undivided leaves, usually soft indumentum with whitish to yellowish setae on basal parts
K. csikii	*K. dinarica* group	(Sub)alpine meadows, tall herb communities	Central W Balkan Peninsula	p	
K. dinarica	*K. dinarica* group	(Sub)alpine meadows, tall herb communities	Central W Balkan Peninsula; Italy	p	
Drymeia Group			Widespread	p	Monopodial growth with a central leaf rosette and lateral flowering shoots
K. drymeia	*K. drymeia* group	Forests and forest margins	Central and SE Europe, N Italy	p	
Longifolia Group			Regional, disjunct	p	Leaves undivided, lanceolate, with entire margins, stem leaves narrowed towards base; basal leaves hairy, stem leaves glabrous; peduncles glandular
K. longifolia	*K. longifolia* group	Upper montane to alpine meadows and tall herb communities	S and E Alps, S and E Carpathians	p	
Midzorensis Group			Regional, continuous	p	Leaves undivided, lanceolate, with entire margins, stem leaves with subcordate basis; basal leaves hairy, stem leaves glabrous; peduncles glandular or eglandular
K. midzorensis	*K. longifolia* group	Tall herb communities	E central Balkan Peninsula	p	
Montana Group			Widespread	m-p	Yellow-flowering, partly tall herbs, with undivided to lyrate leaves; peduncles glandular
K. involucrata	*K. montana* group	Subalpine meadows	Anatolia to Caucasus	p	
K. montana	*K. montana* group	Tall herb communities	Caucasus to Urals	m	
Pancicii Group			Stenoendemic	p	All leaves glabrous, undivided, lanceolate, with entire margins; peduncles eglandular
K. pancicii	*K. longifolia* group	Damp meadows	Zlatibor planina (Serbia)	p	
SW European Group			Widespread, but disjunct	p	Heterogeneous: often undivided, rarely deeply divided (*K. subscaposa*), leaves glabrous to densely hairy
K. basaltica	*K. longifolia* group	(Sub)alpine grasslands on volcanic bedrock	Massif Central (France)	p	
K. godetii	*K. longifolia* group	Wet meadows, fens	Massif Central (France) to Jura Mountains (Switzerland)	p	
K. lebrunii (syn. *K. salvadoris*)	*K. longifolia* group	Tall herb communities, open forests	E Pyrenees	p	
K. subcanescens	*K. subcanescens/ persicina* group	Tall herb communities, (sub)alpine meadows	W Alps	p	
K. subscaposa	-	Dry grasslands	Iberian Peninsula	p	
Xerophytic Group			SW Alps to W Balkans	p	Not or only weakly divided leaves with one to three pairs of lobes and a dense lanuginose to tomentose indumentum
K. albanica	*K. velutina* group	Dry grasslands	Central W Balkan Peninsula	p	
K. calycina	*K. velutina* group	Mountain grasslands	Central and S Italy, Sicily	p	

Table 2 (*continued*)

AFLP groups and constituent species	Ehrendorfers (1962a, 1981) groups	Habitat preference of individual species	Distribution type of AFLP groups and distribution of individual species	Longevity: mono- or polycarpic	Morphological characteristics of AFLP groups
K. collina	*K. arvensis* group	Dry grasslands and subruderal places	S France and NW Italy?	p	
K. illyrica	*K. arvensis* group	Dry grasslands	SE Alps to NW Balkan Peninsula	p	
K. mollis	*K. velutina* group	Mountain grasslands	SW Alps	p	
K. pectinata	*K. dalmatica* group	Dry mountain grasslands	NW Balkan Peninsula (Velebit)	p	
K. purpurea	*K. arvensis* group	Dry grasslands and ruderal places	SW and S Europe	p	
K. travnicensis	*K. fleischmannii* group	Dry mountain grasslands	Central NW Balkan Peninsula	p	
K. velebitica	*K. velutina* group	Dry mountain grasslands	NW Balkan Peninsula	p	
K. velutina	*K. velutina* group	Dry mountain grasslands, rocks	S Alps	p	

flower head and montane to subalpine, open mesic habitats. Our AFLP and sequence data (Fig. 3, Supplementary Figs. S1, S2 and S4) demonstrate that this group is far less homogeneous than suggested. It should be better split up into four smaller groups, represented in our study by (1) *K. pancicii*, (2) *K. midzorensis*, (3) *K. longifolia* and (4) the SW European *K. basaltica*, *K. godetii*, *K. lebrunii*, *K. subcanescens* as well as the morphologically aberrant *K. subscaposa*. Whereas the plastid sequences (Supplementary Fig. S1) only support the isolated position of *K. subscaposa*, the ITS analysis suggests a position of these groups between the two major terminal groups in the NeighbourNet (Fig. 3B, Supplementary Fig. S2). In the AFLP tree (Supplementary Fig. S4) the four groups are loosely linked neighbours, each of them relatively well supported, in the NeighbourNet diagram (Fig. 3A) they all emerge from the same zone, supporting their loose relationships.

Knautia pancicii is a stenoendemic species from wet meadows of the Zlatibor mountain range in southwestern Serbia. Originally suggested as a possible link between *K. longifolia* and *K. midzorensis* (Ehrendorfer, 1962a), it is in fact the only and strongly divergent member of the **Pancicii Group** (Fig. 3, Supplementary Fig. S4). *Knautia longifolia*, the only member of the **Longifolia Group**, has its main distribution in the southeastern Alps and Carpathians, but was also reported from the central Balkan Peninsula (Ehrendorfer, 1981; Fig. 1). We were unable to confirm this latter occurrence despite considerable efforts. It is likely that relevant reports refer to other taxa, e.g. *K. midzorensis* (Diklić, 1973). Although the *K. longifolia* populations from the southeastern Alps form a homogeneous group in the AFLP data, in the ITS NeighbourNet (Fig. 3B) they share a morphologically counterintuitive split with diploid *K. illyrica* from the northern Balkan Peninsula, a relationship resolved with low support (BS 56) also in the ITS phylogeny (Supplementary Fig. S2). Plastid DNA haplotypes of the Longifolia Group were markedly heterogeneous, including three haplotype groups (Supplementary Fig. S3). *Knautia midzorensis* from the mountains of the central Balkan Peninsula (western Stara Planina, Rila, Pirin) was considered diploid (Ehrendorfer, 1962a, 1981), but throughout the distribution range we mostly found tetraploid populations (Frajman et al., unpubl.). Only plants from the Bulgarian Verila-Rui mountain range (K426) and from southeastern Serbia (K215, K271) are diploid and were therefore included in the present study. According to AFLP data they form the maximally supported **Midzorensis Group**, with only loose and unsupported links to the following group and bearing haplotypes of the Red Haplotype Group only (Fig. 3A, Supplementary Fig. S4). The **SW European Group** includes species with undivided leaves from the Jura Mountains (*K. godetii*), the Massif Central (*K. basaltica*) and the eastern Pyrenees (*K. lebrunii* sensu Fl. Iberica: Devesa, 2007), all assembled by Ehrendorfer (1962a, 1981) in his *K. longifolia* group. In addition, this group also includes *K. subcanescens* from the western Alps and the morphologically very different *K. subscaposa* from the Iberian Peninsula. This SW European Group forms a strongly supported lineage in the ITS phylogeny (Supplementary Fig. S2: BS 96, PP 1), appears as a clade without bootstrap support in the AFLP data (Fig. 3A, Supplementary Fig. S4) but is not apparent in the plastid tree (Supplementary Fig. S1). The SW European Group comprises three well-supported AFLP lineages (Fig. 3, Supplementary Fig. S4). The first includes the two genetically divergent species *K. lebrunii* and *K. subcanescens*. Whereas both populations of *K. lebrunii* were diploid, our global sampling of *K. subcanescens* comprised one tetraploid population in accordance with Ehrendorfer (1962a), whereas the two here included populations were diploid (previously unknown; Frajman et al., unpublished). Ehrendorfer (1962a) used the name *K. subcanescens–K. persicina*-group for a supposedly exclusively tetraploid species assembly from the Alps (also comprising *K. baldensis*, *K. norica* and *K. transalpina*). The second lineage consists of *K. basaltica* and *K. godetii*. Although

geographically separated by ca. 350 km and ecologically divergent (the former growing in alpine grasslands on volcanic bedrock and the latter in damp meadows and bogs), both are intermingled in the AFLP and ITS data sets. The third lineage is formed by the geographically distant *K. subscaposa*, which morphologically resembles *K. collina* (sensu Devesa, 2007) from our Xerophytic group (Fig. 3). It exhibits a deviating genome size intermediate between diploids and tetraploids (Frajman et al. unpubl.) indicating reproductive isolation from other taxa, but chromosome counts from Spain (Devesa, 2007) confirm it as diploid.

The geographically most isolated taxa, *K. montana* from the Caucasus and *K. involucrata* from northern Anatolia, constitute Ehrendorfer's (1962a) *K. montana* group but are not closely related to *K. longifolia*, as suggested previously. The **Montana Group** is strongly supported by AFLP data (Fig. 3A, Supplementary Fig. S4). The ITS sequence data (Supplementary Fig. S2) support a separation into two taxa, but the relationships between them and to other members of sect. *Trichera* remain unclear. In spite of their geographical isolation they carry the most frequent and widespread plastid haplotype H25 (Fig. 3, Supplementary Fig. S3).

Our DNA data suggest considerable changes on the informal groups of *K. velutina*, *K. dalmatica*, *K. fleischmannii* and particularly *K. arvensis*, previously based on morphology, eco-geography and ploidy alone (Ehrendorfer, 1962a). Instead, AFLPs (Fig. 3A, Supplementary Fig. S4) and partly also ITS (Fig. 3B, Supplementary Fig. S2) and plastid sequences (Supplementary Figs. S1 and S3) form the basis for the recognition of the following four, strongly modified groups, which better reflect phylogenetic relationships.

Ehrendorfer's (1962a) *K. velutina* group, comprising only diploids with dense and soft indumentum, is largely supported by AFLP data (BS 58), but has to include additional, less hairy taxa (Fig. 3A, Supplementary Fig. S4). In the ITS NeighbourNet (Fig. 3B) the same taxa (with exception of *K. illyrica*) are separated from other accessions by several splits, but their monophyly is not supported (Supplementary Fig. S2). This species cluster is a heterogeneous assemblage of species distributed from the Pyrenees and Sicily over the Apennines to the Alps and the Balkan Peninsula. The shared, albeit not exclusive feature is a preference for xeric habitats, often submediterranean-montane grasslands. Based on its ecology we name this species cluster the **Xerophytic Group**. It includes *K. albanica*, *K. calycina*, *K. mollis*, *K. velebitica*, *K. velutina* (all from Ehrendorfer's *K. velutina* group), accessions of *K. arvensis*, *K. collina*, *K. illyrica*, *K. purpurea* (from Ehrendorfer's *K. arvensis* group), *K. pectinata* (from Ehrendorfer's *K. dalmatica* group) and *K. travnicensis* (from Ehrendorfer's *K. fleischmannii* group), but not *K. carinthiaca* (Fig. 3). Diploid cytotypes of *K. illyrica* and *K. travnicensis* were not known prior to our study (Frajman et al. unpubl.). AFLP data support the links between all these species relatively well but fail to recover interspecific relationships (Fig. 3A, Supplementary Fig. S4). Exceptions are the robust support (BS 88) of *K. mollis* (southwestern Alps) and *K. collina* (southwestern Alps to Pyrenees) as sister species. This pair is the well-supported (BS 87) sister to *K. calycina* sampled in the central Apennines and Sicily (as well as single samples of *K. arvensis* and *K. purpurea* from the northern Apennines). Although previously deemed closely related (Ehrendorfer, 1962a; Szabó, 1911), *K. calycina* and *K. collina* are clearly separated from the *K. arvensis* alliance (Fig. 3). The circumscription of *K. mollis*, *K. collina* and *K. purpurea* in southwestern Europe was considered uncertain as their morphological separation is sometimes difficult due to the presence of transitional variants (Ehrendorfer, 1962a), probably as a result of extensive hybridisation. For instance, transitional forms link *K. collina* at lower elevations with *K. mollis* at higher elevations over a broad altitudinal amplitude in the Italian Alpi Ligure (P. Schönswetter & B. Frajman, pers. field obs.). The inclusion of *K. illyrica* in the Xerophytic Group is supported by AFLPs, whereas ITS data suggest an isolated position and indicate shared splits with *K. longifolia* (Fig. 3; see above). Affinities of *K. illyrica* with *K. arvensis* are refuted, contrary to traditional concepts (Ehrendorfer, 1962a). A close relationship of the Dinaric *K. pectinata* and *K. velebitica* was moderately supported by AFLPs (BS 63), while *K. travnicensis* and *K. albanica* formed separate robust subgroups (BS 86 and BS 96, respectively). The more pronounced divergence of taxa within the Xerophytic Group as opposed to the Southern Arvensis Group is paralleled by plastid data, as three out of four haplotype groups are present in the Xerophytic Group whereas only the most frequent one is present in the Southern Arvensis Group.

The narrow endemic *K. carinthiaca* from the eastern Central Alps is not a member of the Xerophytic Group (Ehrendorfer, 1962a had placed it into his *K. velutina* group), but constitutes a separate maximally supported **Carinthiaca Group** sharing some splits with the Northern and Southern Arvensis Groups in the AFLP data (Fig. 3A), whereas ITS rather suggests an unresolved position close to *K. dinarica*, possibly with introgression from the Southern Arvensis Group (Fig. 3B, Supplementary Fig. S2).

Knautia arvensis and its relatives form a taxonomically dreadful, complex and heterogeneous diploid–tetraploid complex encompassing several closely related species, considered derived within sect. *Trichera* (Ehrendorfer, 1962a). Most are polycarpic perennials, but monocarpic individuals may occur in pure or mixed populations. Frequent hybridisation both within the group and with taxa of other groups is likely one of the key aspects in the evolution of this complex. The geographically most widespread species is *K. arvensis*, whose preference for anthropogenic semiruderal mesophilous grasslands has undoubtedly facilitated dispersal through human activities and thus fostered hybridisation and gene flow with closely related species. A recent study on central European populations has revealed their complex evolution which included isolation in ecologically distinct refugia, repeated colonisation by distinct lineages, hybridisation and recurrent polyploidisation (Kolář et al., 2012). Dedicated analyses based on a more detailed sampling throughout the range of *K. arvensis* will be necessary before it will eventually be possible to draw taxonomic conclusions. Nevertheless, the available AFLP and ITS data (Fig. 3, Supplementary Figs. S2 and S4) allow the clear separation of two groups within the morphologically unequivocal *K. arvensis*: (1) populations from north of the Alps together with *K. slovaca* constitute the Northern Arvensis Group and (2) those from the Alps southwards group together with morphologically similar species to form the Southern Arvensis Group. Only one sample of *K. arvensis* (K331) from the northern Apennine clustered with the Xerophytic Group. The **Northern Arvensis Group** is based so far on the few known diploid *K. arvensis* populations from the northern, western and central Czech Republic (mostly growing on serpentine soils), clearly set aside from all other representatives of the species by AFLP (Fig. 3A, Supplementary Fig. S4) and ITS (Fig. 3B, Supplementary Fig. S2). Both markers also suggest affinities (though not supported) with *K. slovaca*, an endemic of the Western Carpathians.

The **Southern Arvensis Group** is supported by AFLP (Fig. 3A, Supplementary Fig. S4), albeit with low support. In addition to the main bulk of populations from the widely distributed *K. arvensis*, it comprises *K. ambigua*, *K. lucana*, *K. macedonica*, *K. purpurea* and *K. visianii*. A similar assemblage, which includes also accessions of other taxa (*K. csikii*, *K. dinarica*, *K. drymeia*, *K. slovaca*), receives high support in the ITS tree (Supplementary Fig. S2) and is separated by a long split in the ITS NeighbourNet (Fig. 3B). This suggests that more distantly related taxa may have been involved in the formation of the central European *K. arvensis* populations. In the southern and south-eastern Balkan Peninsula *K. arvensis* is apparently replaced by the closely related, invariably monocarpic *K. visianii* as well as by the polycarpic *K. ambigua* and *K. macedonica* (Fig. 1). The latter two are only distinguished by flower colour

(yellow or pale pink in *K. ambigua* vs. dark red in *K. macedonica*), but polychromatic populations with colours ranging from yellow to blackish-red render the separation of these taxa highly questionable (Ehrendorfer, 1962a). Interestingly, the diploid *K. lucana* from southern Italy is nested within the Balkan *K. visianii*, a relationship suggested already by Ehrendorfer (1962a). Similar amphiadriatic disjunctions and close phylogenetic relationships are known also from other plant groups (e.g., *Campanula garganica* group, Frajman and Schneeweiss, 2009; *Euphorbia barrelieri*, Frajman and Schönswetter, unpublished data; Poldini, 1969).

5. Conclusions

Similarly as other groups with rampant hybridisation and polyploidisation (e.g. *Galium*: Ehrendorfer, 1955, 1963 or *Achillea*: Ehrendorfer, 1959; Guo et al., 2008), the genus *Knautia* is notorious for the paucity of reliable morphological characters. Consequently, previous infrasectional classifications or informal groupings of the species-rich section *Trichera* (Ehrendorfer, 1962a; Szabó, 1911) were hampered by the lack of clear morphological circumscriptions of several species due to considerable phenotypic plasticity and high levels of interspecific gene flow. By circumscribing genepools, our AFLP data allow to test whether critical taxa are indeed evolutionary lineages worth of taxonomic recognition (such as *K. pancicii* and *K. subscaposa*) or should rather be combined with other species (e.g., *K. ambigua* and *K. macedonica*). It is evident, however, that the shallow structure unravelled by our molecular data makes a reasonable formal infrasectional classification impossible. Even our informal groups are often difficult to delimit and have limited predictive value. Some unite morphologically fairly different taxa, which either exhibit similar ecological requirements (as the Xerophytic Group) or share geographical provenance (e.g., the SW European Group, the Montana Group or the Southern Arvensis Group).

Even if we cannot offer a ready-to-use taxonomic framework of the genus *Knautia*, the present study provides by far the most comprehensive phylogeny of the genus so far available and sets the stage for unravelling the evolutionary history of the polyploid members of section *Trichera*. Besides, it identifies several key aspects of the genus' evolutionary history at the basal, diploid level, such as young age, massive and rapid range expansions and extensive hybridisation. Gene flow among populations has likely been facilitated both by range contractions and expansions during previous glacial cycles, as well as by the effects of human activity and disturbance over the past centuries. For instance, it appears very likely that *K. arvensis*, the most widespread and abundant member of the genus, has expanded its range dramatically following anthropogenic deforestation. Only then it had the chance to come into contact and hybridise with autochthonous entities from natural habitats such as primary dry or wet meadows or rocky outcrops. The regular presence of intermediates even between morphologically clearly distinct and ecologically separated species such as *K. arvensis* and *K. drymeia* (Ehrendorfer, 1962a; Hayek, 1914; Szabó, 1911; Štěpánek, 1997) is suggestive of almost unlimited gene flow across species boundaries. This latter process was certainly also fostered by the uniformity of flower morphology throughout section *Trichera* and its adaptation to generalist pollinators (Hayek, 1914).

Acknowledgments

The present study was financed by the European Commission in the SEE-ERA.NET PLUS framework (project "Evolution, biodiversity and conservation of indigenous plant species of the Balkan Peninsula" to P.S.) and the Austria–Croatia bilateral project "Evolution of polyploid species on the Balkan Peninsula". Collecting permits were issued for the territory of Croatia (532-08-01-01/3-11-02 to A. Alegro), for the Parco Nazionale del Gran Sasso e Monti della Laga (UT-RAU-SCNZ 384 to S. Bogdanović) and for the Parco Nazionale della Majella (6318/3 to S. Bogdanović). Our thanks go to all collectors listed in Supplementary Table S1 for samples and to M. Magauer, D. Pirkebner and M. Winkler for their excellent laboratory work. We are most grateful to P. Daniel Schlorhaufer and his colleagues from the Botanical Gardens of the University of Innsbruck for successfully cultivating our living collection of *Knautia*. The curators Ernst Vitek (W) and Walter Till (WU) allowed us to sample herbarium specimens of *K. byzantina* and *K. orientalis*.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2014.01.028.

References

Albach, D.C., Martínez-Ortega, M.M., Chase, M.W., 2004. *Veronica*: parallel morphological evolution and phylogeography in the Mediterranean. Plant Syst. Evol. 246, 177–194.
Altekar, G., Dwarkadas, S., Huelsenbeck, J.P., Ronquist, F., 2004. Parallel metropolis-coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. Bioinformatics 20, 407–415.
Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.
Arrigo, N., Tuszynski, J.W., Ehrich, D., Gerdes, T., Alvarez, N., 2009. Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. BMC Bioinformatics. http://dx.doi.org/10.1186/1471-2105-10-33.
Avino, M., Tortoriello, G., Caputo, P., 2009. A phylogenetic analysis of Dipsacaceae based on four DNA regions. Plant Syst. Evol. 279, 69–86.
Bergsten, J., 2005. A review of long-branch attraction. Cladistics 21, 163–193.
Bonin, A., Bellemain, E., Bronken Eidesen, P., Pompanon, F., Brochmann, C., Taberlet, P., 2004. How to track and assess genotyping errors in population genetics studies. Mol. Ecol. 13, 3261–3273.
Bryant, D., Moulton, V., 2004. Neighbor-net: an agglomerative method for the construction of phylogenetic networks. Mol. Biol. Evol. 21, 255–265.
Caputo, P., Cozzolino, S., Moretti, A., 2004. Molecular phylogenetics of Dipsacaceae reveals parallel trends in seed dispersal syndromes. Plant Syst. Evol. 246, 163–175.
Carlson, S.E., Mayer, V., Donoghue, M.J., 2009. Phylogenetic relationships, taxonomy, and morphological evolution in Dipsacaceae (Dipsacales) inferred by DNA sequence data. Taxon 58, 1075–1091.
Carlson, S.E., Linder, H.P., Donoghue, M.J., 2012. The historical biogeography of *Scabiosa* (Dipsacaceae): implications for Old World plant disjunctions. J. Biogeogr. 39, 1086–1100.
Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Mol. Ecol. 9, 1657–1659.
Devesa, J.A., 2007. *Knautia* L. In: Devesa, J.A., Gonzalo, R., Herrero, A. (Eds.), Flora Iberica. Plantas vasculares de la Península Ibérica e Islas Baleares, vol. XV, Rubiaceae–Dipsacaceae. Real Jardín Botánico, CSIC, Madrid, pp. 286–305.
Diklić, N., 1973. Dipsacaceae B. Juss. In: Josifović, M. (Ed.), Flora SR Srbije, vol. 5. Srpska Akademija Nauka i Umetnosti, Beograd, pp. 536–584.
Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.
Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2011. Geneious v5.4. <http://www.geneious.com/>.
Drummond, C.S., Eastwood, R.J., Miotto, S.T., Hughes, C.E., 2012. Multiple continental radiations and correlates of diversification in *Lupinus* (Leguminosae): testing for key innovation with incomplete taxon sampling. Syst. Biol. 61, 443–460.
Ehrendorfer, F., 1955. Hybridogene Merkmalsintrogression zwischen *Galium rubrum* L. s.str. und *G. pumilum* Murr. s.str. Österr. Bot. Z. 102, 195–234.
Ehrendorfer, F., 1959. Differentiation-hybridization cycles and polyploidy in *Achillea*. Cold Spring Harbor Symp. Quant. Biol. 24, 141–152.
Ehrendorfer, F., 1962a. Beiträge zur Phylogenie der Gattung *Knautia* (Dipsacaceae), I. Cytologische Grundlagen und allgemeine Hinweise. Österr. Bot. Z. 109, 276–343.
Ehrendorfer, F., 1962b. Cytotaxonomische Beiträge zur Genese der mitteleuropäischen Flora und Vegetation. Ber. Deutsch. Bot. Ges. 75, 137–152.
Ehrendorfer, F., 1963. In: Sylvester-Bradley, P.C. (Ed.), Post-Tertiary speciation in Europe: sect. Lepto-Galium. Nature 199, 129.
Ehrendorfer, F., 1976. *Knautia* L.. In: Tutin, T., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A. (Eds.), Flora Europaea, vol. 4. Cambridge University Press, Cambridge, pp. 60–67.

Ehrendorfer, F., 1981. Neue Beiträge zur Karyosystematik und Evolution der Gattung *Knautia* (Dipsacaceae) in den Balkanländern. Bot. Jahrb. Syst. 102, 225–238.

Ehrendorfer, F., 1982. *Knautia*. In: Pignatti, S. (Ed.), Flora d'Italia, vol. 2. Edagricole, Bologna, pp. 664–670.

Frajman, B., Schneeweiss, G.M., 2009. A Campanulaceous fate: the Albanian stenoendemic *Asyneuma comosiforme* in fact belongs to isophyllous *Campanula*. Syst. Bot. 34, 595–601.

Frajman, B., Schönswetter, P., 2011. Giants and dwarfs: molecular phylogenies reveal multiple origins of annual spurges within *Euphorbia* subg. *Esula*. Mol. Phylogen. Evol. 61, 413–424.

Frajman, B., Eggens, F., Oxelman, B., 2009. Hybrid origins and homoploid reticulate evolution within *Heliosperma* (Sileneae, Caryophyllaceae) – a multigene phylogenetic approach with relative dating. Syst. Biol. 58, 328–345.

Fuertes Aguilar, J., Nieto Feliner, G., 2002. Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). Mol. Phylogen. Evol. 28, 430–447.

Guo, Y.-P., Saukel, J., Ehrendorfer, F., 2008. AFLP trees versus scatter plots: evolution and phylogeography of the polyploid complex *Achillea millefolium* agg. (Asteraceae). Taxon 57, 153–169.

Gutiérrez Larena, B., Fuertes Aguilar, J., Nieto Feliner, G., 2002. Glacial-induced altitudinal migrations in *Armeria* (Plumbaginaceae) inferred from patterns of chloroplast DNA haplotype sharing. Mol. Ecol. 11, 1965–1974.

Hayek, A., 1914. *Knautia*. In: Hegi, G. (Ed.), Illustrierte Flora von Mitteleuropa, vol. VI/1. A. Pichler's Witwe & Sohn, Wien, pp. 290–303.

Hong, D., Ma, L., Barrie, F.R., 2011. *Pterocephalus* Vaillant ex Adanson. In: Al-Shehbaz, I.A., Bartholomew, B., Boufford, D.E., Brach, A.R., Shilong, C., Gilbert, M.G., Deyuan, H., Qiming, H., Jérémie, J., Kress, W.J., Dezhu, L., McNamara, W.A., Peng, C., Raven, P.H., Simpson, D.A., Turland, N.J., Watson, M.F., Zhengyi, W., Bing, X., Qiner, Y., Libing, Z., Xianchun, Z. (Eds.), Fl. China 19. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis, MO, pp. 655–656.

Hudson, R.R., Coyne, J.A., 2002. Mathematical consequences of the genealogical species concept. Evolution 56, 1557–1565.

Huson, D.H., 1998. SplitsTree: a program for analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.

Jabbour, F., Renner, S.S., 2012. A phylogeny of Delphinieae (Ranunculaceae) shows that *Aconitum* is nested within *Delphinium* and that Late Miocene transitions to long life cycles in the Himalayas and Southwest China coincide with bursts in diversification. Mol. Phylogen. Evol. 62, 928–942.

Kolář, F., Fér, T., Štech, M., Trávníček, T., Dušková, E., Schönswetter, P., Suda, J., 2012. Bringing together evolution on serpentine and polyploidy: spatiotemporal history of the ecologically differentiated diploid-tetraploid complex of *Knautia arvensis* (Dipsacaceae). PLoS One 7, e39988.

Maddison, W.P., Maddison, D.R., 2010. Mesquite: a modular system for evolutionary analysis. Version 2.73. <http://mesquiteproject.org>.

Matthews, V.A., 1972. *Knautia* L. In: Davis, P.H. (Ed.), Flora of Turkey and East Aegean Islands, 4. University Press, Edinburgh, pp. 598–601.

Mayer, V., Ehrendorfer, F., 2013. The phylogenetic position of *Pterocephalidium* and the new African genus *Pterothamnus* within an improved classification of Dipsacaceae. Taxon 62, 112–126.

Mitchell-Olds, T., 2001. *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. Trends Ecol. Evol. 16, 693–700.

Müller, K., Müller, J., 2003. QuickAlign: a new alignment editor. Plant Mol. Biol. Rep. 21, 5.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76, 5269–5273.

Nylander, J.A.A., 2004. MrAIC.pl. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Poldini, L., 1969. Kritische Bemerkungen über die *Euphorbia saxatilis-triflora-kerneri* Verwandtschaft. Acta Bot. Croat. 28, 317–328.

R Development Core Team, 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing Vienna, Austria. <http://www.R-project.org>.

Rebernig, C.A., Weiss-Schneeweiss, H., Schneeweiss, G.M., Schönswetter, P., Obermayer, R., Villasenor, J.L., Stuessy, T.F., 2010. Quaternary range dynamics and polyploid evolution in an arid brushland plant species (*Melampodium cinereum*, Asteraceae). Mol. Phylogen. Evol. 54, 594–606.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Schaal, B.A., Olsen, K.M., 2000. Gene genealogies and population variation in plants. Proc. Natl. Acad. Sci. U.S.A. 97, 7024–7029.

Schaefer, H., Hechenleitner, P., Santos-Guerra, A., de Sequeira, M.M., Pennington, R.T., Kenicer, G., Carine, M.A., 2012. Systematics, biogeography, and character evolution of the legume tribe Fabeae with special focus on the middle-Atlantic island lineages. BMC Evol. Biol. 12, 250.

Schneeweiss, G.M., Schönswetter, P., Kelso, S., Niklfeld, H., 2004. Complex biogeographic patterns in *Androsace* (Primulaceae) and related genera: evidence from phylogenetic analyses of nuclear ITS and plastid trnL-F sequences. Syst. Biol. 53, 856–876.

Schönswetter, P., Tribsch, A., Niklfeld, H., 2002. Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* Sternb. & Hoppe (Campanulaceae) in the European Alps. Mol. Ecol. 11, 2637–2647.

Schönswetter, P., Solstad, H., Escobar García, P., Elven, R., 2009. A combined molecular and morphological approach to the taxonomically intricate European mountain plant *Papaver alpinum* s.l. (Papaveraceae). Taxa or informal phylogeographical groups? Taxon 17, 1326–1343.

Shaw, J., Lickey, E.B., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E., Small, R.L., 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am. J. Bot. 92, 142–166.

Smith, S.A., Donoghue, M.J., 2008. Rates of molecular evolution are linked to life history in flowering plants. Science 322, 86–89.

Stearns, S.C., 1992. The evolution of life histories. Oxford University Press, Oxford, UK.

Stebbins, G.L., 1957. Self fertilization and population variability in the higher plants. Am. Nat. 91, 337–354.

Štěpánek, J., 1983. Eine neue Art der Gattung *Knautia* (Dipsacaceae) aus Westkarpaten. Preslia 55, 1–8.

Štěpánek, J., 1997. *Knautia* L.. In: Slavík, B. (Ed.), Kvtena České Republiky 5. Academia, Praha, pp. 543–554.

Sun, Y., Skinner, D.Z., Liang, G.H., Hulbert, S.H., 1994. Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor. Appl. Genet. 89, 26–32.

Swofford, D.L., 2002. PAUP. Phylogenetic Analysis Using Parsimony (∗and Other Methods), ver. 4.0 Beta 10. Sinauer Associates, Sunderland.

Szabó, Z., 1911. A *Knautia* génusz monographiája. Kiadja a Magyar tudományos academia, Budapest.

Tank, D.C., Olmstead, R.G., 2008. From annuals to perennials: phylogeny of subtribe *Castillejinae* (Orobanchaceae). Am. J. Bot. 95, 608–625.

Van de Peer, Y., De Wachter, R., 1997. Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. Comput. Appl. Biosci. 13, 227–230.

Verlaque, R., 1985–1986. Étude biosystématique et phylogénétique des Dipsacaceae. II–IV. Rev. Cytol. Biol. Veg. Botaniste 8, 117–168, 171–243; 9, 5–72, 97–176.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van De-Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23, 4407–4414.

Willyard, A., Cronn, R., Liston, A., 2009. Reticulate evolution and incomplete lineage sorting among the ponderosa pines. Mol. Phylogen. Evol. 52, 498–511.

Wood, T.E., Takebayashi, N., Barker, M.S., Mayrose, I., Greenspoon, P.B., Rieseberg, L.H., 2009. The frequency of polyploid speciation in vascular plants. Proc. Natl. Acad. Sci. U.S.A. 106, 13875–13879.

Yogeeswaran, K., Frary, A., York, T.L., Amenta, A., Lesser, A.H., Nasrallah, J.B., Tanksley, S.D., Nasrallah, M.E., 2005. Comparative genome analyses of *Arabidopsis* spp.: Inferring chromosomal rearrangement events in the evolutionary history of *A. thaliana*. Genome Res. 15, 505–515.

Young, T.P., Augspurger, C.K., 1991. Ecology and evolution of long-lived semelparous plants. Trends Ecol. Evol. 6, 285–289.

Yu, Y., Than, C., Degnan, J.H., Nakhleh, L., 2011. Coalescent histories on phylogenetic networks and detection of hybridisation despite incomplete lineage sorting. Syst. Biol. 60, 138–149.