Molecular evidence for reticulate speciation in *Astragalus* (Fabaceae) as revealed by a case study from section *Dissitiflori*

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Abstract

Although hybridisation has long been recognised as a major force driving speciation in land plants, it has not been evidenced yet in *Astragalus*, the largest angiosperm genus. Here we reveal the possible contribution of hybridisation to speciation in *Astragalus* by employing cloning of the nrDNA ITS region and sampling three plastid regions (*ycf*1, *ndh*F-*rpl*32 and *rpl*32-*trn*L) in taxa belonging to section *Dissitiflori*. Phylogenetic network and tree analyses uncovered various levels of intra-individual/intraspecific polymorphism of ITS in most taxa investigated. Two distantly related ribotype groups were found to be shared by the closely related polyploids *Astragalus pallescens*, *A. peterfii* and *A. pseudoglaucus* suggesting ancient hybridisation followed by incomplete lineage sorting (i.e. shared ancestral polymorphism) in nrDNA ITS. Reticulation is invoked as an underlying evolutionary process also behind the statistically highly supported incongruent placement of *A. pseudoglaucus* and *A. vesicarius* subsp. *pastellianus* in nuclear vs. plastid phylogenies. The phylogenetic results also shed light on taxonomic controversies in the section, such as the false synonimisation of *A. peterfii* under *A. vesicarius* s.l. Our results provide evidence for the (at least past) existence of speciation processes driven by hybridisation in *Astragalus*.

Key words: hybridisation, *ycf*1, nrDNA ITS cloning, allopolyploidy, reticulation

Introduction

Astragalus L. is the most species-rich plant genus among angiosperms with ca. 2400 species (Lock and Schrire 2005). The genus is distributed in all continents, excluding Australia, and has its major centres of diversification in southwest and central Asia, the Sino-Himalayan region, western North America and in the Andes in South America (Lock and Schrire 2005). Together with more than 40 other genera, *Astragalus* belongs to the IRLC (inverted repeat-lacking clade) of Fabaceae which is characterised by the loss of a 25 kb long inverted repeat in the chloroplast genome (Wojciechowski *et al.* 1999).

Chromosome number evolution in *Astragalus* is marked by a split between New vs. Old World taxa. New World *Astragalus* species are almost exclusively aneuploids with a most frequent basic chromosome number n=11, and polyploidy is virtually absent among them [reviewed in Spellenberg (1976) and Wojciechowski *et al.* (1993)]. The situation is markedly different in Old World species, where, besides the widespread diploid chromosome number 2n=16, frequent counts of 2n=32, 48, 64 have been reported by cytological studies (Ledingham 1960; Ledingham and Rever 1963; Fedorov 1969; Philippov *et al.* 2008; Masoud *et al.* 2009). Ploidy levels might be phylogenetically determined, as observed by shared ploidy in closely related species. Accordingly, within the monophyletic (Riahi *et al.* 2011) sect. *Caprini*, all species and subspecies investigated up to date have a base chromosome number of 2n=16 (Podlech 1988; Martin et al. 2008; Sytin 2009). Within the tragacanthic *Astragalus* (a gum producing group having thorny cushion-forming habit), diploid, tetraploid and hexaploid levels have been revealed so far (Masoud *et al.* 2009) which might be consistent with the paraphyly of this group (Kazempour Osaloo *et al.* 2003). In addition to the ploidy levels mentioned until now, dodecaploidy (2n=96) has been uncovered in four species of sect. *Dissitiflori* (Philippov *et al.* 2008). The 2n = ca. 160 in the Romanian endemic *A. roemeri* Simonk. (sect. *Onobrychoidei*) is probably the highest count ever reported

(Ledingham and Rever 1963). The above account indicates a significant role of polyploidy in the evolution of Old World *Astragalus*. Polyploidy, however, has often been coupled with hybridisation throughout the diversification of land plants, making allopolyploidisation one of the most important speciation processes (Grant 1981; Soltis and Soltis 2009).

In spite of the frequent polyploidy in the genus *Astragalus*, hybridisation and allopolyploidy have never been clearly documented for the genus. Moreover, hybridisation is often regarded as being extremely rare, 'exceptional', or non-existing in this legume genus (Podlech 1988; Liston 1992; Judd *et al.* 2008; Kazemi *et al.* 2009). This might be related to the breeding biology of the species. Podlech (1988) hypothesised that autogamy should be the general breeding system in *Astragalus* because in most species the stigma stands between the simultaneously ripening anthers (Barneby 1964) and the pollen falls frequently into the keel of the young flowers with closed petals. Studies dealing with the breeding biology and pollinator types of certain *Astragalus* species, however, are numerous. A review of these, assessing information from 29 taxa of different distribution and taxonomic range, revealed that half of the investigated species were self-compatible, one third were obligate outcrossers whilst the remaining species were self-compatible where outcrossing was beneficial (Watrous and Cane 2011). The main pollinator species (if known) were bees from different genera. The above authors also concluded that predicting the breeding biology of a single *Astragalus* species is speculative owing to the diversity of breeding biologies, lack of correlates with life history or ecology and the general lack of knowledge about most species within this genus.

Evidence suggesting exceptional cases of hybridisation in the genus *Astragalus* exist but are sparse and are based only on morphological observations or results of biochemical analyses. In the Flora of the [former] USSR a few examples of hybridisation are mentioned, such as hybrids between *A. sewertzowii* Bunge and *A. schanginianus* Pall., or between *A. sewertzowii* and *A. lasiopetalus* Bunge (Borisova *et al.* 1946), all of them belonging currently to sect.

Caprini (Podlech 2011). According to Deml (1972), hybrids exist among species of section *Aegacantha*.

Section *Dissitiflori* has about 160 species (Podlech 2011) and is one of the largest sections of the genus. The section is most diverse in Central-Asia (Borisova *et al.* 1946), but its range extends across all Eurasia (Ghahremani-Nejad 2004). In Europe, it comprises some 50 species with mostly disjunct distribution ranges. Section *Dissitiflori* was established by A.P. de Candolle in 1825. Later, Bunge (1868) introduced the same section under the name of *Xiphidium*, unaware that this had already been done by de Candolle (Ranjbar 2004). The lectotype of this section is *Astragalus varius* (Podlech 1990). Species of section *Dissitiflori* are perennials covered with bifurcate or more rarely subbasifixed hairs. In fact, section *Dissitiflori* is the largest bifurcate haired *Astragalus* section (Ranjbar 2004). Species of this section are morphologically characterised by imparipinnate leaves, loose racemes and shortly pedicellate flowers. The flower in sect. *Dissitiflori* has glabrous petals and tubular calyx while the legumes are two-locular with leathery valves (Bunge 1868).

Section *Dissitiflori* seems to provide more evidence of hybridisation in *Astragalus* when compared with other sections. Accordingly, Sytin (1999) hypothesised that hybridisation might have occurred between *A. brachylobus* D.C. and *A. varius* S.G.Gmel.. Podlech (2008) mentions that in Romania there are probably transitions from *A. vesicarius* L. to *A. albicaulis* D.C.. Studying the genetic variability of *A. peterfii* Jáv. using biochemical analysis, Borza (1998) found a pattern of isoenzymes suggesting an allopolyploid origin of this species. The octaploid (2n=64) *A. peterfii* is a strict endemic of the Transylvanian Lowland (Câmpia Transilvaniei, Romania), and is an emblematic species for nature conservation, being known only from two localities.

Due to the widespread polyploidy, morphology-based suggestions of hybridisation, as well as the putative allopolyploid origin of *A. peterfii*, sect. *Dissitiflori* represents a promising system

where the relative contribution of hybridisation to the diversification of species is worth exploring. At the same time, some taxa whose names emerge in the context of hybridisation in *Astragalus*, have controversial taxonomic status. For instance, *A. peterfii*, itself, has been synonymised by Pânzaru (2006) with *A. vesicarius* subsp. *pastellianus* (Pollini) Arcang., an endemic northern Italian subspecies with *locus classicus* in Italy, near Verona. Two other taxa, *A. pseudoglaucus* Klokov and *A. tarchankitucus* Boriss., were also treated as 'perhaps to be included' under *A. vesicarius* subsp. *pastellianus* in Flora Europaea (Chater 1968). Later, the new combination *A. vesicarius* subsp. *pseudoglaucus* (Klokov) Ciocârlan was introduced (Ciocârlan and Sârbu 2001). More recently, Podlech (2011) treated *A. pseudoglaucus* as synonym of *A. vesicarius*, and *A. tarchankuticus* as synonym of *A. albicaulis* in his Thesaurus Astragalorum. This account confirms the interest in the problem of origin and relationships of *A. peterfii*, as well as the taxonomic status of several putatively related taxa.

The study of hybrid (reticulate) speciation in plants involves the use of biparentally inherited nuclear markers. Hybrid and allopolyploid species can be identified if copies of a nuclear region originating from different putative parental species are retrieved from an organism and those copies were evolutionary diverged prior to hybridisation. If such copies originating from the maternal parent are homogenised towards the paternal copy-type – a phenomena commonly occurring in case of the internal transcribed spacer region of nuclear ribosomal DNA (Álvarez and Wendel 2003) – inconsistencies between the nuclear and plastid DNA phylogenies may still serve as evidence for reticulate (hybrid) speciation, or eventually allopolyploidisation. Incongruence between nuclear and plastid DNA phylogenies (or paralogy of any particular nuclear loci), however, can also be caused by incomplete lineage sorting and intra-genomic recombination (Rieseberg and Brunsfeld 1992; Xu *et al.* 2012). Discriminating reticulation from incomplete lineage sorting (i.e. retention of ancestral polymorphism) of a given marker is often difficult and represents a challenging task for

evolutionary biologists (Wendel and Doyle 1998; Willyard *et al.* 2009). Nonetheless, the more recent the diversification is, the more likely incomplete lineage sorting can be accounted for shared polymorphism between lineages (Sang 2002).

In this study, we employ nuclear and plastid marker systems to selected members of section *Dissitiflori* in order to (1) test for the presence of hybrid (reticulate) speciation in the genus *Astragalus*, and (2) shed light on the taxonomy of a putative species complex including *A. peterfii*, *A. pseudoglaucus*, *A. tarchankuticus* and subspecies of *A. vesicarius*.

Materials and methods

Taxon sampling

Sampling design focussed on *Astragalus peterfii* because of the allopolyploid origin already suggested for this species based on biochemical analyses (Borza 1998). *Astragalus peterfii* presented a substantial morphological overlap with *A. pallescens* in previous multivariate analysis of morphological characters (Bartha *et al.* 2012a) arguing for the inclusion of the latter into the analyses. The traditionally accepted subspecies of *A. vesicarius* – including samples from their *locus classicus* (Table 1) – have also been included in order to determine their relationships with *A. pseudoglaucus*, *A. tarchankuticus* and *A. peterfii*. Additional species from section *Dissitiflori* growing in Romania or in the neighbouring countries were also included. These were *A. albicaulis*, *A. asper* Jack., *A. ucrainicus* Popov and *A. varius*. Given that we had their samples, they were included to add taxonomic information within the section. Moreover, the inclusion of *A. varius* was warranted by its type species status for sect. *Dissitiflori*. Acquiring material of additional species is ongoing. Among the taxa included in this study, *Astragalus asper* has been traditionally classified under the monotypic section *Pedina*; however, it has been transferred recently into section *Dissitiflori* (Podlech 2011). For phylogenetic tree reconstruction, *A. glycyphyllos* L. (section *Glycyphyllus*) was selected as outgroup because previous molecular analyses confirmed that this section is outside of sect. *Dissitiflori* (Kazempour Osaloo *et al.* 2003).

Plant material and DNA extraction

We used both herbarium and field collected material for DNA extraction (Table 1). In the latter case, leaves were dried and stored in silica gel until extraction. In case of one sample of *Astragalus vesicarius* subsp. *pastellianus*, DNA was purchased from DNA bank (Botanical Garden, Berlin-Dahlem). ZR Plant/Seed DNA Kit (Zymo Research) was used for genomic DNA extraction. At least two specimens per species were included in the nuclear DNA

analysis, while for analyses of plastid DNA regions one specimen was sequenced for each species.

PCR amplification

Primers obtained from the literature as well as newly designed ones (Table 2) were used for PCR-amplification of the target DNA regions. For amplification of the nuclear ribosomal internal transcribed spacer region (ITS1-5.8S-ITS2) the reaction mixture contained 0.2 volume 5× Green GoTaq Flexi Buffer (Promega), 0.2 mM each of dNTPs (Promega), 2 mM $MgCl_2$, 0.2 μM of each primer, 1.25 U GoTaq DNA Polymerase (Promega) and approximately100 ng genomic DNA (per reaction). The thermal cycler conditions for nrITS amplification were as follows: initial denaturation step at 94°C for 2 min, followed by 33 cycles of denaturation at 94°C for 50 sec, annealing at 51°C for 30 sec and extension at 72°C for 1 min; the thermal cycling was ended by a final extension of 72°C for 5 min. The PCR protocol for plastid regions was similar to that used for nrITS amplification except for the following differences: 3 mM $MgCl_2$ was added instead of 2 mM and 0.4 μM primer was added instead of 0.2 μM. The amplification and sequencing of an approx. 1.5 kb portion from the 3' end of the hypothetical plastid open reading frame 1 (*ycf*1) was performed as described in Bartha *et al.* (2012b). The original primers of Shaw *et al.* (2007) for amplifying the *ndh*F-*rpl*32 and *rpl*32-*trn*L intergenic spacer regions (IGS) performed poorly in our target species, but the partial sequences obtained with these primers were sufficient for designing new *Astragalus*-specific forward and reverse primers (Table 2). Amplification of *ndh*F-*rpl*32 was achieved using the following PCR regimen: initial denaturation step at 94°C for 4 min, 40 cycles of denaturation at 94°C for 45 sec, annealing at 61°C for 1 min and extension at 72°C for 1 min, followed by a final extension step of 5 min at 72°C. The PCR regimen for *rpl*32-*trn*L was the same as that for *ndh*F-*rpl*32 except for the primer annealing temperature (54°C). All amplifications were performed using a Gradient Palm-Cycler.

Sequencing and cloning

The Wizard SV Gel and PCR Clean-Up System (Promega) was used to purify PCR products. Sequencing of nrITS followed three strategies: (1) direct sequencing of those samples which provided clear (i.e. double-peak lacking, full-length readable) electropherograms (*A. vesicarius* subsp. *vesicarius* and *A. vesicarius* subsp. *carniolicus*); (2) cloning of nrITS in case of lower quality (but still readable) chromatograms resulting from direct sequencing of *A. asper*, *A. varius*, *A. vesicarius* subsp. *pastellianus* and *A. ucrainicus* accessions; (3) cloning of nrITS from the rest of species for which direct sequencing efforts resulted in unreadable sequences and/or chromatograms containing unambiguous double peaks which are indicative of intra-individual sequence polymorphism (Nieto Feliner and Roselló 2007). Cloning was performed using pGEM-T Vector System I (Promega) for ligation and GeneJET Plasmid Miniprep Kit (Fermentas) for plasmid isolation. nrITS was cloned from 23 out of the 25 accessions investigated. Numbers of clones per specimens used in the nrITS analyses are shown in Table 1, whereas the total number of clones analysed per species is summarised in Table 3. For most taxa 10–20 clones were sequenced, however for octaploid species *A. peterfii* 55 clones were sequenced to the raise possibility of recovering additional paralogs. Direct nrITS sequences were obtained using primer ITS5 (White *et al.* 1990), nrITS clones were sequenced using the universal primer M13, while direct sequencing of the plastid regions was achieved with the primers mentioned in Table 2. DNA sequencing was performed by a commercial service (Macrogen Inc., South Korea). GenBank accession numbers for *ycf*1, *ndh*F-*rpl*32, *rpl*32-*trn*L regions and nrITS direct sequences are listed in Table 1, while Appendix A1 contains the accession numbers for nrITS clone sequences. One *A. glycyphyllos* nrITS clone sequence (used for outgroup) was downloaded from GenBank (Table 1).

Phylogenetic analysis

Sequences were directly exported from the chromatograms to FASTA format files using ChromasLite v.2.01 (Technelysium Pty). *ycf*1 fragments of the same sample (obtained with different internal sequencing primers) were assembled using BioEdit (Hall 1999). Sequences were aligned in MEGA5 (Tamura *et al.* 2011) by algorithm ClustalW followed by manual adjustment. If a certain column of the nrITS alignment (containing 183 sequences) had only one singleton mutation (a nucleotide variant appearing only once among the sequences) this was considered as PCR error and was replaced by the major nucleotide type of the column. nrITS clone sequences with uncommonly long deletions and/or many point mutations were regarded as pseudogenes and were excluded from subsequent work. To search for recombinant sequences (i.e. PCR chimeras) in the nrITS dataset, the Phi test (Bruen *et al.* 2006) was performed as implemented in SplitsTree v.4.10 (Huson and Bryant 2006).

Because of the large number of clones and presence of diverged nrITS paralogs in some of the samples, a phylogenetic network approach rather than a hierarchical tree-based one was at first used in the nrITS analysis. The program Collapse v.1.2 (Posada 2006) was used for defining unique sequence types (ribotypes) in the nrITS dataset and assessing the distribution of these ribotypes within and between accessions. The defined unique ribotypes were then included in parsimony network analysis, as implemented in TCS (Clement *et al.* 2000), in order to delimitate putative groupings (ribotype groups) and assess genealogical relationships amongst them. Gaps were treated as a fifth state in the TCS analysis since there were only single base long (potentially parsimony informative) indels in the matrix of aligned ribotypes.

As a next step, phylogenetic tree reconstruction methods (see below) were applied to the nrITS dataset in order to substantiate the result obtained with the TCS analysis and to assess confidence to groupings.

To check for the possible discrepancy between the three plastid datasets, the incongruence length difference (ILD) test (Farris *et al.* 1994) as implemented in *PAUP** 4.0b10 (Swofford

2002) was employed using 100 replicates of heuristic searches with tree bisection-reconnection (TBR) branch-swapping. Since no statistically supported incongruence (p=0.25) was found between the different plastid regions, they were combined into a single dataset (hereafter referred to as 'plastid dataset' to which phylogenetic tree analyses were applied).

Phylogenetic tree analyses employed for both plastid and nuclear datasets included maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML).

Maximum parsimony analysis was run in *PAUP** and relied on heuristic search using 1000 random addition of sequence replicates and TBR branch swapping with MULTREES option in effect, MAXTREES set to 15,000 (without possibility of increasing the tree buffer) and a limit of ten trees retained for each iteration step. Characters were weighted equally and gaps were treated as missing data. The statistical robustness of tree branches was estimated via bootstrapping; 1000 pseudo-replicates were performed in *PAUP** with MAXTREES re-set to 1000 and with the retention of one tree per replicate. Bootstrap support (BS) values were considered as low/weak (50–74%), moderate (75–84%) and strong (85–100%).

MrModeltest v2. (Nylander 2004) was used to select the nucleotide substitution models for the three plastid and the nrITS DNA regions using the Akaike Information Criterion (AIC). Bayesian analysis was carried out on a partitioned plastid dataset with the models listed in Table 4, while in case of the nuclear dataset the GTR+I model was used. Bayesian analysis involved two simultaneous runs of 4,000,000 generations of Monte Carlo Markov chains by saving every one hundredth tree. Each run employed four simultaneous chains. After checking convergence in Tracer (Drummond and Rambaut 2007), i.e. effective sample sizes (ESS) were >1000, a 50% majority-rule consensus phylogram was generated in MrBayes with a 'burn-in' of 10,000 trees (25%). Clades were considered according to the following criteria: well supported or strongly supported, 99–100% posterior probability (PP); moderately supported, 95–98% PP; and not supported, below 95% PP.

Maximum likelihood (ML) analysis relied on RAxML (Stamatakis 2006) using the RAxML GUI version 1.2 (Silvestro and Michalak 2011) under the GTR + Γ model of sequence evolution (as recommended by the RAxML manual). Nodal support values for the ML topology were estimated using the rapid bootstrap algorithm implemented in RAXML employing 100 replicates (Stamatakis *et al.* 2008).

Results

DNA alignments

Sequence statistics (and assessment of MP heuristic searches) for the plastid dataset are summarised in Table 4.

The nrITS alignment matrix contained 181 cloned and two direct sequences, 598 characters and 56 variable sites. The phi test did not find statistically significant (p=0.0323) evidence for the presence of chimeric sequences in the nrITS data matrix. Collapse retrieved 54 ribotypes from the original 183 sequences. Variable nucleotide positions from the ribotype alignment are shown in Supplementary Table S1. Only one base long, potentially parsimony informative indels were found at three positions in the ribotype matrix. The nrITS sequence alignment used for phylogenetic tree reconstruction included 85 sequences: 84 from ingroup and one of *A. glycyphyllos* as outgroup. The strategy regarding nrITS sequence selection for phylogenetic tree reconstruction was to include all species, subspecies and accessions and all ribotypes within these (i.e. include those 82 nrITS clone and two direct sequences for which GenBank accession numbers (Appendix A1, Table 1) were obtained). This strategy seemed to be a 'middle course' between including all of the 183 sequences (would have contained many identical sequences) or including only the 54 unique ribotypes (would not have reflected the full intra-individual nrITS polymorphism on a phylogenetic tree). The above strategy resulted in an alignment containing 607 characters and 74 variable sites.

Data matrices are available upon request from the corresponding author.

Phylogenetic tree analysis of the plastid dataset

Topologies inferred by the three phylogenetic tree reconstruction methods are broadly congruent (Fig. 1). *Astragalus asper* diverges first in the phylogenies. This species is followed by a dichotomy leading to the two main clades in the trees. Within one of these clades *A. ucrainicus* is branching first. Next comes the highly supported (MP BS 90%, BI PP 100%,

ML BS 96%) clade A which is split into two highly supported though unresolved polytomies: one encompassing *A. albicaulis*, *A. pseudoglaucus* and *A. vesicarius* subsp. *pastellianus*, the another comprising *A. pallescens*, *A. peterfii* and *A. tarchankuticus*.

Minor topological discrepancies between the single most parsimonious tree, the Bayesian consensus phylogram and the best maximum likelihood tree are restricted to relationships within clade B and are not supported statistically (Fig. 1) Parsimony analysis recovered *A. varius* as branching first within clade B followed by *A. vesicarius* subsp. *carniolicus* and a highly supported grade comprising the rest of *A. vesicarius* subspecies. In the Bayesian tree *A. vesicarius* subsp. *carniolicus* forms a polytomy with *A. varius* and another branch leading to the highly supported dichotomy of the remaining *A. vesicarius* subspecies. Contrary to this, in the ML tree (not shown) *A. varius* forms an unsupported dichotomy with *A. vesicarius* subsp. *carniolicus* and this is sister with the previously know well supported subclade of clade B. The taxonomic coverage of clade B, however, is consistent; moreover, clade B itself is highly supported by different analyses (MP BS 99%, BI PP 100%, ML BS 99%). Unlike *A. vesicarius* subsp. *pastellianus*, which is nested within one of the subclades of clade A, the rest of *A. vesicarius* subspecies (along with *A. varius*) are found within clade B. This division (clade A plus *A. ucrainicus* versus clade B) is apparently reflected by the petal colour of species: members of the former have whitish to yellow coloured petals, whereas species of clade B have purplish to violet flowers (in *A. vesicarius* subsp. *albidus* the wings and keel are whitish). It would be premature, however, to attribute importance to this morphological division since only a small fraction of the European species of section *Dissitiflori* have been included in the analyses.

Parsimony network analysis of nrITS sequences

Analyses using the software Collapse revealed which sequences were identical within accessions (Appendix A1) and which ribotypes were shared by different specimens and species, information which was incorporated into the parsimony network construction.

The TCS analysis reconstructed two unconnected networks at 95% connection limit (Fig. 2). The smaller network containing less ribotypes, was referred to as ribotype group A, while within the bigger network containing most of the ribotypes six more ribotype groups (B–G) could be separated (Fig. 2, Table 3).

Cloned nrITS sequences retrieved from *A. asper*, *A. ucrainicus* and *A. vesicarius* each belonged to one single ribotype group (C, G, and E, respectively), while sequences of the rest of the five species clustered into 2–4 different groups. *Astragalus peterfii* has the single highest number of ribotype groups identified within it; nonetheless, a higher number of clones were sequenced in this species.

The number of different species which shared the same ribotype group varied from two to four. Sequences from ribotype groups C and F belonged exclusively to one species (*A. asper*, and *A. varius*, respectively) while the rest of the ribotype groups were shared by different species. Interestingly, groups A and B are the dominant ribotype groups of *A. pallescens*, *A. peterfii* and *A. pseudoglaucus*.

The frequency of nrITS sequences belonging to a given ribotype group for each species has shown considerable differences (Table 3). This led to the recognition that certain ribotype groups could be associated with given species, e.g. ribotype group E could be considered the 'vesicarius-like' ribogroup because it included sequences mainly from *A. vesicarius*.

The TCS network reflects every shared ribotype at the level of accessions (Fig. 2). The most common ribotype within ribotype group A occurred in both accessions of *A. pallescens* and *A. tarchankuticus* and all of the four accessions of *A. peterfii*, whereas this type of sequence was retrieved only from one *A. pseudoglaucus* accession. The most frequent (central) ribotype

from group B occurred in both accessions of *A. pseudoglaucus*, three out of four accessions of *A. peterfii* and one out of the two accessions of *A. pallescens*. Four clones of the *A. varius* accession from Romania differed in a single base duplication from the six clones of the *A. varius* accession from Hungary (Fig. 2, Supplementary Table S1). The most intricate distribution of ribotypes among the accessions of one species is shown by *A. peterfii*: two of its clones belonging to group D were recovered, one from one accession from the *locus classicus*, and one from the population of Căianu. The second accession of *A. peterfii* (pet7) from the latter location, however, did not contain sequences characteristic for group D, but had clones belonging to the 'vesicarius-type' ribogroup.

Phylogenetic tree analysis of the nrITS dataset

Maximum likelihood analysis of 85 nrITS sequences retrieved all of the groupings found in the previous network analysis (Fig. 2, Fig. 3). The corresponding clades (A–G) were denominated according to these groupings. Heuristic search of the same dataset resulted in 2088 equally most parsimonious trees with 122 steps in length (tree not shown), their strict consensus tree also recovering the previously identified groupings. With the exception of clade E these clades were recovered also by the Bayesian analysis (tree not shown). The ML, MP and Bayesian trees were broadly congruent not only with regard to tree topology but also clade confidence (Fig. 3). The most relevant differences in topology and clade support between the ML, Bayesian and MP trees are: (i) clade B was resolved as sister to clade C on the ML tree with strong support (BS=95%). This sister relationship was recovered – though unsupported (PP=80%) – on the Bayesian tree but was collapsed on the strict consensus of most parsimonious trees; (ii) clade G was resolved as sister to the 'D-E-F' clade on the ML and Bayesian trees with weak BS (58%) and unsupported PP (66%) and this sister relationship was collapsed into a basal polytomy on the MP strict consensus tree; (iii) clade F was recovered by all type of analyses but gained moderate support (BS=76%) on ML tree,

week support on MP tree (BS=61%) and was unsupported on the Bayesian tree (PP=94%); (iv) clade E was collapsed in the Bayesian tree but was recovered and gained weak support in both ML and MP analyses. The rest of denominated clades, however, were at least moderately but in most cases well supported (Fig. 3).

Both analyses identified clade A as diverging first, implying that sequences of e.g. *A. peterfii* from clade A are more closely related (or in several cases identical) to sequences of *A. pallescens* and *A. pseudoglaucus* from the same clade than to sequences of *A. peterfii* from clade B.

As seen in the network analyses shown above, *A. vesicarius* nrITS sequences were exclusively nested in clade E, all *A. asper* sequences in clade C and all *A. ucrainicus* sequences in clade G. Most of *A. pallescens*, *A. peterfii* and *A. pseudoglaucus* sequences are confined to clades A and B. Clade F includes only *A. varius* sequences but *A. varius* is present also in clade E. In three cases presence of a certain taxon in a group was represented by a sequence (or sequences) originating from one taxonomic sample which made the tree (and the corresponding network) result more indecisive. These were: *A. tarchankuticus* in group G, *A. varius* in group E and *A. peterfii* in group E.

Discussion

Reticulation in Astragalus section Dissitiflori

The co-existence of phylogenetically distinct (and even distant) ribotypes within the same individual and species (i.e. intra-individual/intraspecific paralogy of nrITS) suggests that evolutionary processes such as merging (different from dichotomous splitting of lineages) took place in section *Dissitiflori*. Although for phylogenetic inference, nrITS has to be handled carefully (Álvarez and Wendel 2003; Nieto Feliner and Roselló 2007), with the results from the plastid phylogeny, nrITS sequence analysis and the clone data (i.e. from the frequency distribution of ribotypes) some conclusions can be drawn on the phylogeny of the target species.

In our view, the findings related to the paralogy of nrITS can be best explained by reticulation and/or incomplete lineage sorting. The presence of the same ribotype groups (A and B) in *A. peterfii*, *A. pallescens* and *A. pseudoglaucus* (Table 3, Figures 2 and 3) can refer to the merging of A and B lineages either in each of these species separately or in their common ancestor. The former would presume repeated reticulate events while the latter refers to a more ancient hybridisation followed by incomplete lineage sorting during subsequent speciation. As reflected by the ML phylogram of nrITS sequences (Fig. 3), a striking divergence exists between the A and B clades, each encompassing separately closely related, or identical sequences of *A. pallescens*, *A. peterfii* and *A. pseudoglaucus* [in case of the well supported (ML 99% BS, MP 80% BS, BI 100% PP) clade B] or sequences of *A. pallescens*, *A. peterfii*, *A. pseudoglaucus* and *A. tarchankuticus* [in case of the well supported (ML 93% BS, MP 98% BS, BI 100% PP) clade A]. The nrITS phylogram together with TCS network of ribotypes, therefore, reflects the sharing of distantly related ribotypes by closely related species which may favour the scenario of a more ancient hybridisation and retention of ancestral polymorphism (‘deep coalescence’) during speciation. According to our current

knowledge, distribution ranges of the above three species do not overlap but are geographically close or even adjacent to each other: *A. peterfii* grows in the Transylvanian basin, *A. pseudoglaucus* is confined to the north-western part of Black Sea region, whereas the area of *A. pallescens* stretches from Southern Ukraine to Southern Russia. Distribution range of a putative 'proto-*Astragalus*' species harbouring nrITS lineages A and B could have overlapped with the extant disjunct distribution of these species, where they might have locally diversified.

A parental species status of the more widespread *A. pallescens* (2n=32) for *A. peterfii* and/or *A. pseudoglaucus* (2n=64) at the western edge of the first species' range (and therefore 'more recent' speciation events) cannot be ruled out but this might not affect the hypothesis of a'more ancient' merging of A and B lineages. A BLAST search of sequences 'gla2.4' and 'gla2.1' (central ribotypes of groups A and B) against the rather comprehensive *Astragalus* nrITS sequence collection of GenBank (but without the sequences generated in the present study) found *A. pseudorhacodes* Gontsch. [AB051979 (sect. *Macrocystodes*)] and *A. xiphidioides* Freyn & Sint. [AB051976 (sect. *Dissitiflori*)] most similar to sequence 'gla2.4' and *A. neurophyllus* Franch. and *A. xanthomeloides* Korovin & Popov [AB231121 and AB231095 (sect. *Macrocystis*)] most similar to sequence 'gla2.1'. Both sections *Macrocystis* and *Macrocystodes*, as well as *A. xiphidioides*, are endemic to Central Asia which suggests that the center of diversification of sect. *Dissitiflori* cannot be circumvented when pinpointing the geographic origin of A and B lineages. Whether these lineages persist independently in separate (parental) species also in the studied region will hopefully be revealed by further sampling.

The occurrence of sequences belonging to 'vesicarius ribogroup E' in genomes of *A. albicaulis*, *A. peterfii* and *A. varius* (i.e. presence of these species in clade E of nrITS phylogeny) may demonstrate a past or extant introgressive potential of *A. vesicarius* and

argues for hybridisation. We do not have information on the breeding biology of our species of interest which do not permit definite conclusions regarding the source of paralogy. Even if these species are obligate selfers at present, they or their ancestor could have hybridised in the past. The genome of several perennial polyploid soybean species (*Glycine* subgenus *Glycine*), for instance, bears evidence of reticulate evolution although it is reproducing nowadays predominantly by selfing (Doyle *et al.* 2004).

In spite that some major contributions to the phylogeny of *Astragalus* and its sister genus *Oxytropis* are based partially or totally on nrITS (Wojciechowski et al. 1993; Wojciechowski et al. 1999; Kazempour Osaloo et al. 2003, 2005; Kazemi et al. 2009; Archambault and Strömvik 2012; Javanmardi et al. 2012), these studies do not report the cloning of this marker. Although the presence of possible paralogous copies were checked in *Astragalus* as part of a large study in Rosaceae (Campbell *et al.* (1997), no evidence was found for the presence of paralogs in two Old World and two New World *Astragalus* species (M. Wojciechowski, *personal communication* (2012)), thus, the present study can be considered to be the first work reporting serious paralogy of nrITS in a group of *Astragalus* by utilising extensive cloning. Scherson *et al.* (2005) screened novel nuclear loci for reconstructing phylogenies at low taxonomic levels in New World (Neo-) *Astragalus*. They confirmed by cloning the presence of different copies of two nuclear loci (ARG10 and FENR) and SNPs in the nuclear locus tRALS in some taxa of New World *Astragalus*. This pattern, however, was interpreted as a consequence of duplication events, and presence of alleles at the given loci without phylogenetic significance. Therefore, our interpretation for the presence of paralogy in the nrITS in *Astragalus* is the first invoking reticulation as the possible source of paralogy into consideration. Moreover, the reticulate structure of nrITS in the polyploid *A. pallescens* [2n=32 (Philippov *et al.* 2008)], *A. peterfii* [2n=64 (Ledingham and Rever 1963)] and *A. pseudoglaucus* [2n=64 (Pavlova and Kozhuharov (1993), under *A. glaucus*)] is suggestive for

their allopolyploid origin, thus allopolyploidy in *Astragalus* is evidenced here for the first time.

Along with the paralogy of nrITS uncovered in most of the studied species, hybridisation is also suggested by at least two instances of contrasting discrepancies between plastid and nuclear phylogenies. Incongruence in placement of certain *Astragalus* species on nuclear and plastid trees has been reported by Kazempour Osaloo *et al.* (2003) and Kazemi *et al.* (2009). These authors interpreted this phenomenon as being most likely caused by long-branch attraction, since all conflicting species were placed on long branches and they were not linked morphologically. Additionally, these authors emphasised the extreme rarity or lack of hybridisation in the genus as another argument supporting their interpretation on the origin of incongruence. Topological incongruence between plastid (Fig. 1) and nuclear (Fig. 3) trees in our study clearly involve *A. pseudoglaucus* and *A. vesicarius* subsp. *pastellianus*, and are apparently not the result of long branch attraction. *Astragalus pseudoglaucus* is closely related to *A. pallescens, A. peterfii* and *A. tarchankuticus* (see clade A on nrITS phylogram), and to *A. pallescens* and *A. peterfii* (clade B on nrITS phylogram). In the plastid phylogeny, however, *A. pseudoglaucus* is resolved as sister to *A. albicaulis* and *A. vesicarius* subsp. *pastellianus* (Fig. 1).

As for the second example, all *A. vesicarius* nrITS sequences (incl. *A. vesicarius* subsp. *pastellianus*) are nested in clade E of the nuclear phylogeny, which is sister to clade F encompassing most of *A. varius* sequences (Fig. 3). This might be concordant with clade B of plastid phylogeny including *A. varius* and all *A. vesicarius* subspecies but *A. vesicarius* subsp. *pastellianus*. The incongruent placement of the latter subspecies is confirmed by its deep and statistically highly supported embedding in clade A of the plastid phylogeny.

A plausible explanation for the unanticipated, but at the same time highly supported, place of both *A. pseudoglaucus* and *A. vesicarius* subsp. *pastellianus* on the plastid phylogeny could

be provided by chloroplast capture (Rieseberg and Brunsfeld 1992) implying introgression of the *pseudoglaucus-pastellianus* plastid lineage into species having totally different nrITS structure. A second hypothesis illustrating topological discrepancies of these two species could be explained by nuclear gene flow followed by concerted evolution of nrITS. In order to pull apart the relative contribution of these biological phenomena to the establishment of present genetic structure, the use of low-copy nuclear gene markers is necessary which are at least less susceptible to concerted evolution (Zimmer *et al.* 1980; Hillis *et al.* 1991). Low-copy nuclear genes will hopefully also reconcile the differences in frequency distribution of nrITS ribotypes among different accessions of *A. peterfii*. These differences and the overall pattern presented in Table 3 could partially be explained also by random clone selection, the stochasticity of PCR, and primers preferentially picking up one ribotype group.

Incomplete concerted evolution of nrITS

The parallel persistence of the dominant ribotype groups A and B in a single genome suggests a retarded concerted evolution (Campbell *et al.* 1997) of nrITS. The A and B copies might persist in the putative parental progenitors or in their descendants forming a unique ribotype group (a single group per species, according to the present concept). Retardation or incompletion of concerted evolution has long been known in other plant groups (both within diploids and polyploids). Classical examples for incomplete concerted evolution with respect to the nuclear ribosomal DNA includes *Amelanchier* (Campbell *et al.* 1997), *Arabidopsis suecica* (O'Kane *et al.* 1996), *Brassica napus* (Bennett and Smith 1991), *Paeonia* (Sang *et al.* 1995) but new examples are continuously being discovered and – according to Liu *et al.* (2006) – incomplete homogenization of nrITS is the rule rather than the exception. Factors such as the presence of different nrITS arrays on different chromosomes (e.g. due to allopolyploidy), asexual reproduction, and perennial habit (Sang *et al.* 1995; Campbell *et al.*

1997) may promote the maintenance of nrITS polymorphism [i.e. mitigation of unequal crossing over and gene conversion to complete concerted evolution (Hillis *et al.* 1991)].

Taxonomic conclusions

The presence of specific ribotype-groups combined with the plastid phylogeny permit some taxonomic conclusions. For instance, all the traditionally accepted subspecies of *A. vesicarius* have nrITS sequences exclusively in ribotype group E (Table 3); nrITS sequences of *A. pseudoglaucus* and *A. pallescens* are found mostly within groups A and B, but the latter two taxa differ in their placement on the plastid phylogeny. We conclude that our results do not support the synonymisation of *A. peterfii* under *A. vesicarius* subsp. *pastellianus* as proposed by Pânzaru (2006). This, however, does not refute the fact that *A. vesicarius* might have contributed to the formation of our species of interest since ribotype group E (in a small portion) was recovered also from *A. peterfii*.

A similar situation exists between *A. pseudoglaucus* and *A. tarchankuticus*: neither the former nor the latter should be included in *A. vesicarius* subsp. *pastellianus*, contrary to Chater (1968). Similarly, our data do not support the submerge of *A. pseudoglaucus* into *A. vesicarius* [as proposed by Ciocârlan and Sârbu (2001) or Podlech (2011)]. The taxonomic independence of *A. tarchankuticus* is also warranted as compared with *A. albicaulis*; the former has ribotype clustered in groups A, D and G, while the latter has ribotypes in groups E and G. Several factors could have led to the uncertain taxonomy of these species. Among the already revealed reticulation, parallelism in morphology might have also hampered the identification of hidden taxonomic richness in this group.

The treatment of *A. vesicarius* subsp. *albidus* as synonym of *A. vesicarius* subsp. *vesicarius* (Podlech 2008) was not reconsidered in the present study. This will require molecular markers with more resolving power.

Another consequence of ribotype group differentiation is the recognition of a species complex within section *Dissitiflori*, formed by *A. pallescens*, *A. peterfii*, *A. pseudoglaucus*, and *A. tarchankuticus*. With the exception of *A. tarchankuticus* all of these species are reported to be polyploids and have ribotype group A as a presumed 'core' of their nrITS array.

Prospect in use of ycf*1 and* ndh*F*-trn*L in* Astragalus

The plastid regions *ycf*1, *ndh*F-*rpl*32 and *rpl*32-*trn*L have been used in *Astragalus* phylogenetics in this study for the first time. Our primary goal in utilising these markers was to infer relationships at the intrasectional level. Their variability, however, is hardly comparable with the already used plastid markers utilised in other sections, namely *trn*T-Y, *trn*S-G and *psb*A-*trn*H in sect. *Caprini* (Riahi *et al.* 2011) and *mat*K, *trn*T-*trn*Y and *trn*H-*psb*A in sect. *Alupecuroidei* (Javanmardi *et al.* 2012) or *ycf*6-*trn*C and *trn*C-*rpo*B in three taxa of Neo-Astragalus (Sokoloff and Gillespie 2011). Rihai *et al.* (2011) suggested rapid and/or very recent diversification of species as argument for the low resolution they obtained within sect. *Caprini*. In spite of the fact that *ycf*1+*ndh*F-*rpl*32+*rpl*32-*trn*L could not discriminate morphologically well diverged species like *A. tarchankuticus* from *A. peterfii*, it was sufficiently variable to delineate several well supported clades within the phylogeny of an even small number of species. The recent comparison of Dong *et al.* (2012) involving 23 plastid regions (among others, *ycf*1 and *rpl*32-*trn*L, but omitting *ndh*F-*rpl*32) found *ycf*1 the most variable followed by *trn*K and *rpl*32-*trn*L, thus confirming the perspectives of these regions in plant phylogenetic inference. In our opinion, the three plastid regions used here might have potential for further phylogenetic studies in *Astragalus*, e.g. for sectional delimitations.

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Table 1. Voucher information for the samples used in the study. In the case of the samples for which no herbarium voucher specimen is available the name of collector, georeferenced locality and date of collection is provided. GenBank accession numbers are listed for nrITS direct, *ycf*1, *ndh*F-*rpl*32 and *rpl*32-*trn*L sequences, whereas acronyms refer to the cloned nrITS samples with the number of analysed clone per sample in parenthesis.

Table 2. List of primers used in this study.

Table 3. Summary statistics for the nuclear marker sequencing: frequency distribution of cloned and direct nrITS sequences within the species and ribotype groups A-G.

Table 4. Sequence statistics of the three plastid regions, results of MP heuristic searches, and the evolutionary models selected under AIC.

Taxon	Location of collection	Type of plant material [a] / Voucher or the name of collector, date and georeferenced locality of collection	nrITS	plastid		
				*ycf*1	*ndh*F-*rpl*32	*rpl*32-*trn*L
A. albicaulis D.C.	Ru: Rostov-on-Don: Belokovylnij	hb / Shishlova 2007 (RWBG)	alb4 (5)	HQ241832	JX464234	JX464245
”	Ua: Luhansk: Svatove	hb / Barbarich 1939 (BP 544290)	alb5 (6)			
A. asper Jack.	Hu: Fejér: Csősz	sg / Gábor Sramkó, 15.06.2010 N 47.051221° E 18.460225°	asp2 (7)			
”	Ro: Cluj: Sucutard	sg / László Bartha, 11.11.2009 N 46.908083° E 24.042000°	asp3 (5)	HQ241833	JX464236	JX464247
A. pallescens M.Bieb.	Ua: Kherson: Sofiivka	sg / Bartha *et al.* 2009 (CL 662376)	pal2 (9)	HQ241835	JX464238	JX464249
”	Ua: Crimea: Verkhn'osadove	sg / Pavel Evseenkov, 10.08.2009 N 44.691633° E 33.712889°	pal4 (6)			
A. peterfii Jáv.	Ro: Cluj: Suatu	sg / László Bartha, 17.05.2009 N 46.793426° E 23.961422°	pet4 (8), pet6 (20)			
”	Ro: Cluj: Căianu	sg / Bartha *et al.* 2009 (CL 662380)	pet5 (9), pet7 (18)	HQ241837	JX464240	JX464251
A. pseudoglaucus Klokov	Ro: Tulcea: Babadag	sg / Bartha *et al.* 2009 (CL 662381)	gla2 (10)			
”	Ro: Tulcea: Jurilovca	sg / Bartha *et al.* 2011 (CL 662386)	gla3 (9)	HQ241834	JX464241	JX464252
A. tarchankuticus Boriss.	Ua: Crimea: Artemivka	sg / Bartha *et al.* 2009 (CL 662379)	tar1 (9)	HQ241838	JX464242	JX464253
”	Ua: Crimea: Olenivka	sg / Bartha *et al.* 2009 (CL 662373)	tar2 (10)			
A. ucrainicus Popov and Klokov	Ua: Crimea: Novoozerne	sg / László Bartha, 13.05.2009 N 45.378722° E 33.116861°	ucr1 (9)			
”	Ua: Crimea: Olenivka	sg / László Bartha, 13.05.2009 N 45.418948° E 32.498408°	ucr2 (4)	HQ241839	JX464243	JX464254
A. varius S.G.Gmelin	Ro: Tulcea: Agigea	sg / Bartha & Marosi 2010 (CL 662399)	var1 (4)	HQ241840	JX464244	JX464255
”	Hu: Bács-Kiskun: Bugac	hb / Siroki 1973 (DE 2076)	var2 (8)			
A. vesicarius subsp. *albidus* (Waldst. & Kit.) Kozhuharov & Pavlova	Ro: Cluj: Apahida	sg / László Bartha, 7.05.2009 N 46.834611° E 23.733806°	ves3 (5)			
”	Ro: Alba: Ormeniş	sg / Alexandru S. Bădărău, 23.05.2009 N 46.379028° E 23.741861°	ves4 (5)	HQ396892	JX464235	JX464246
A. vesicarius subsp. *carniolicus* (A.Kerner) Chater	Sl: Vipava: Nanos	sg / Radalj & Surina 2011 (NHMR 1614)	KC565846	KC565848	KC565849	KC565851

A. vesicarius subsp. *pastellianus* (Pollini) Arcang.	It: Lasa: Allitz	sg / Zippel (DB 1234)	pas2 (6)			
”	It: Friuli: Gemona	hb / Merxmüller & Lippert 1975 (M 29832)	pas3 (5)			
”	It: Veneto: Ceraino	sg / Roberta Salmaso, 18.05.2009. N 45.56900° E 10.83227°	pas5 (4)	HQ241836	JX464239	JX464250
A. vesicarius L. subsp. *vesicarius*	Fr: Provence-Alpes-Côte d'Azur: Briançon	sg / Rolland Douzet, 6.07.2011 N 44.907050° E 6.657344°	KC565847	JQ801569 [b]	KC565850	KC565852
A. glycyphyllos L.	Ro: Covasna: Bixad	sg / Gábor Sramkó, 22.08.2011 N 46.08432° E 25.907487°	AM943382 [c]	HQ241831	JX464237	JX464248

Note: blanks mean samples not analysed for the given marker. Acronyms of vouchers refer to herbarium codes, except for ‘DB’ which denotes the The Berlin-Dahlem DNA Bank.
[a] hb=herbarium, sg=silicagel dried, [b] retrieved from Bartha *et al.* (2012b), [c] downloaded from GenBank.

DNA region	Primer name	Sequence (5'–3')	Used for[a]	Source
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	A/S	(White *et al.* 1990)
	ITS4	TCCTCCGCTTATTGATATGC	A/S	(White *et al.* 1990)
*ycf*1	ycf1-F	ATCMATGGACAARTTGGTT	A	(Bartha *et al.* 2012b)
	ycf1-R	CTAATCGATAATTTGGCC	A	(Bartha *et al.* 2012b)
	IntF1	AAAGGAGCAAACGAAGAAGC	S	(Bartha *et al.* 2012b)
	IntR	TCGTTGAGGTAGTTATTTCG	S	(Bartha *et al.* 2012b)
*ndh*F-*rpl*32	A-ndhF-F[b]	CTCTTTTGTAARGGGTTCGGT	A/S	This study
	A-rpl32-R[c]	CGAGCTAATTSAGATTATTGC	A	This study
*rpl*32-*trn*L	A-rpl32-F[d]	TTGGAAAAAAAAAGGGATATTGG	A/S	This study
	A-trnL(UAG)-R	TCGAACCGAGATGCTCTAGC	A/S	This study

[a] A=amplification, S=sequencing.

[b] anneals to the beginning of the *ndh*F-*rpl*32 intergenic spacer (not to the gene *ndh*F).

[c] anneals to the beginning of the *rpl*32-*trn*L intergenic spacer (not to the gene *rpl*32).

[d] reverse complement of 'rpl32-R' by Shaw *et al.* (2007).

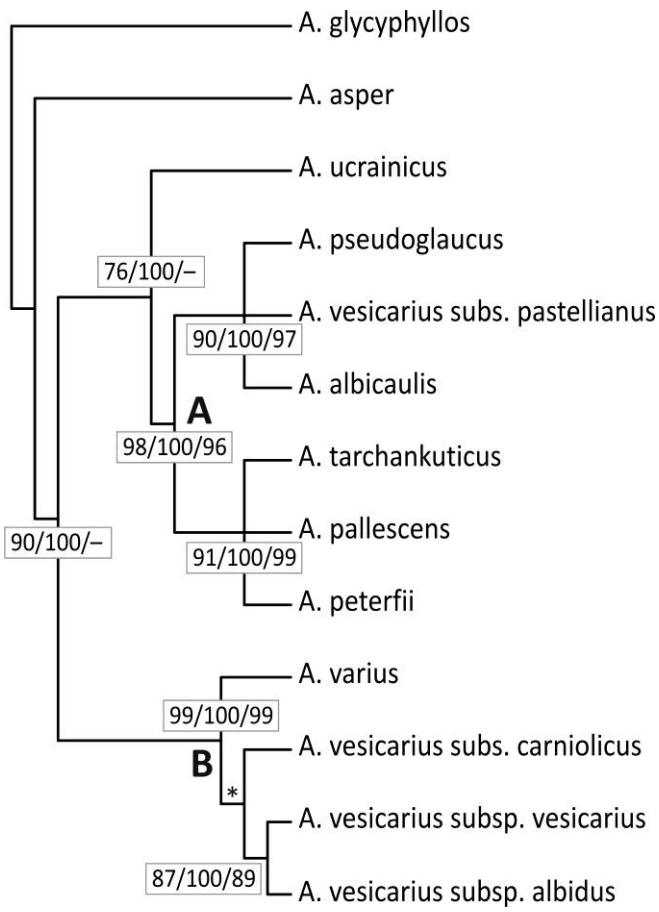
Taxon	No. of individuals analysed	Total no. of sequenced clones per species	No. of direct sequences per species	No. of clones and direct sequences per group						
				A	B	C	D	E	F	G
A. vesicarius subsp. *albidus*	2	10	-	-	-	-	-	10	-	-
A. vesicarius subsp. *pastellianus*	3	15	-	-	-	-	-	15	-	-
A. vesicarius subsp. *carniolicus*	1	-	1	-	-	-	-	1	-	-
A. vesicarius subsp. *vesicarius*	1	-	1	-	-	-	-	1	-	-
A. asper	2	12	-	-	-	12	-	-	-	-
A. varius	2	12	-	-	-	-	-	1	11	-
A. ucrainicus	2	13	-	-	-	-	-	-	-	13
A. albicaulis	2	11	-	-	-	-	-	2	-	9
A. tarchankuticus	2	19	-	9	-	-	9	-	-	1
A. pseudoglaucus	2	19	-	7	12	-	-	-	-	-
A. pallescens	2	15	-	10	5	-	-	-	-	-
A. peterfii	4	55	-	39	10	-	2	4	-	-

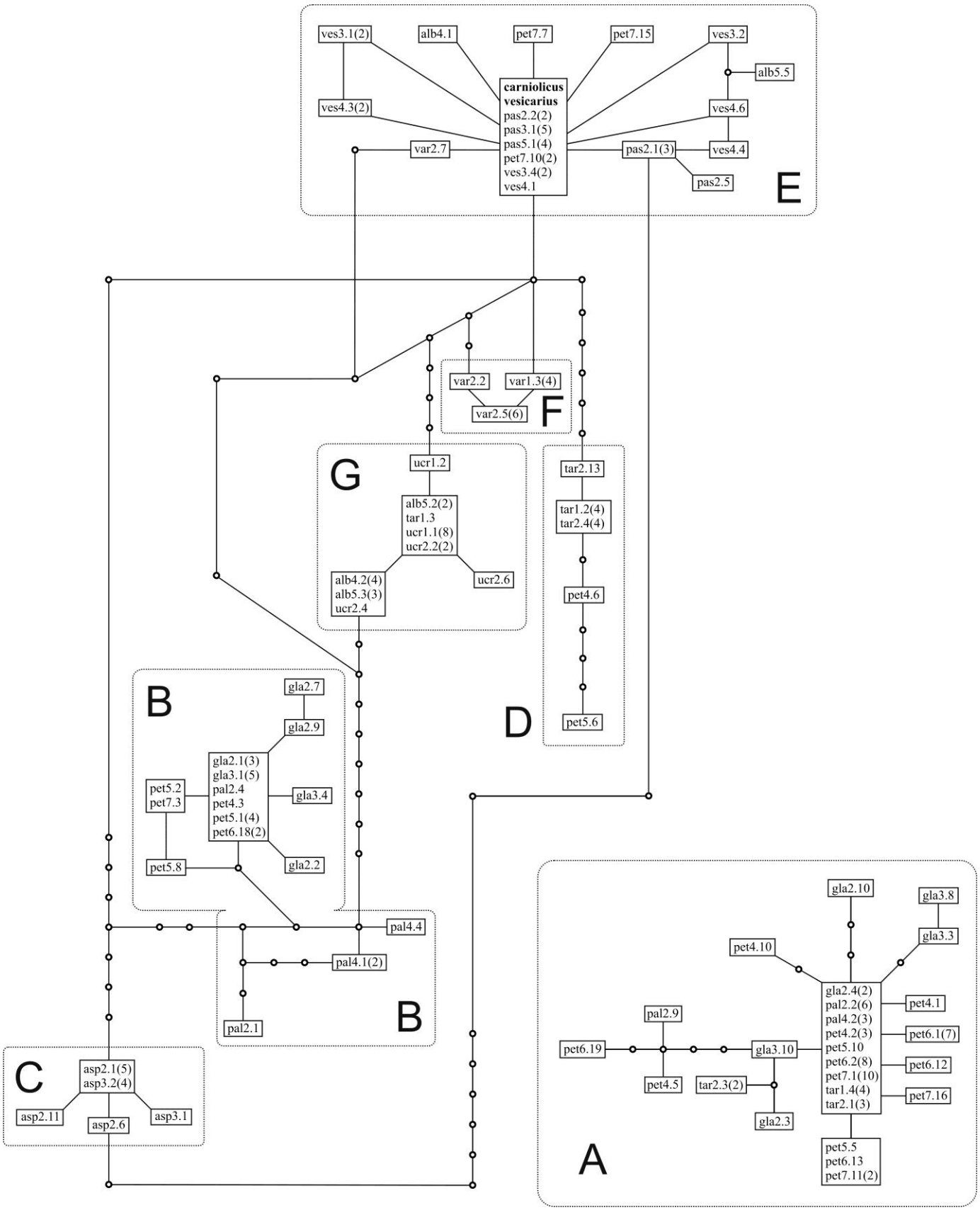
	*ycf*1	*ndh*F-*rpl*32	*rpl*32-*trn*L	combined
alignment length	1514	758	859	3131
no. of variable characters (outgroup included)	95	34	31	160
no. of variable characters (outgroup excluded)	44	19	20	83
no. of parsimony informative characters (outgroup included)	17	8	7	32
no. of parsimony informative characters (outgroup excluded)	15	6	6	27
no. of equally most parsimonious trees	7	3	1	1
CI	0.934	1.0000	0.9697	0.9432
RI	0.8793	1.0000	0.95	0.8980
evolutionary model	GTR+I	GTR	GTR	GTR+I/GTR/GTR

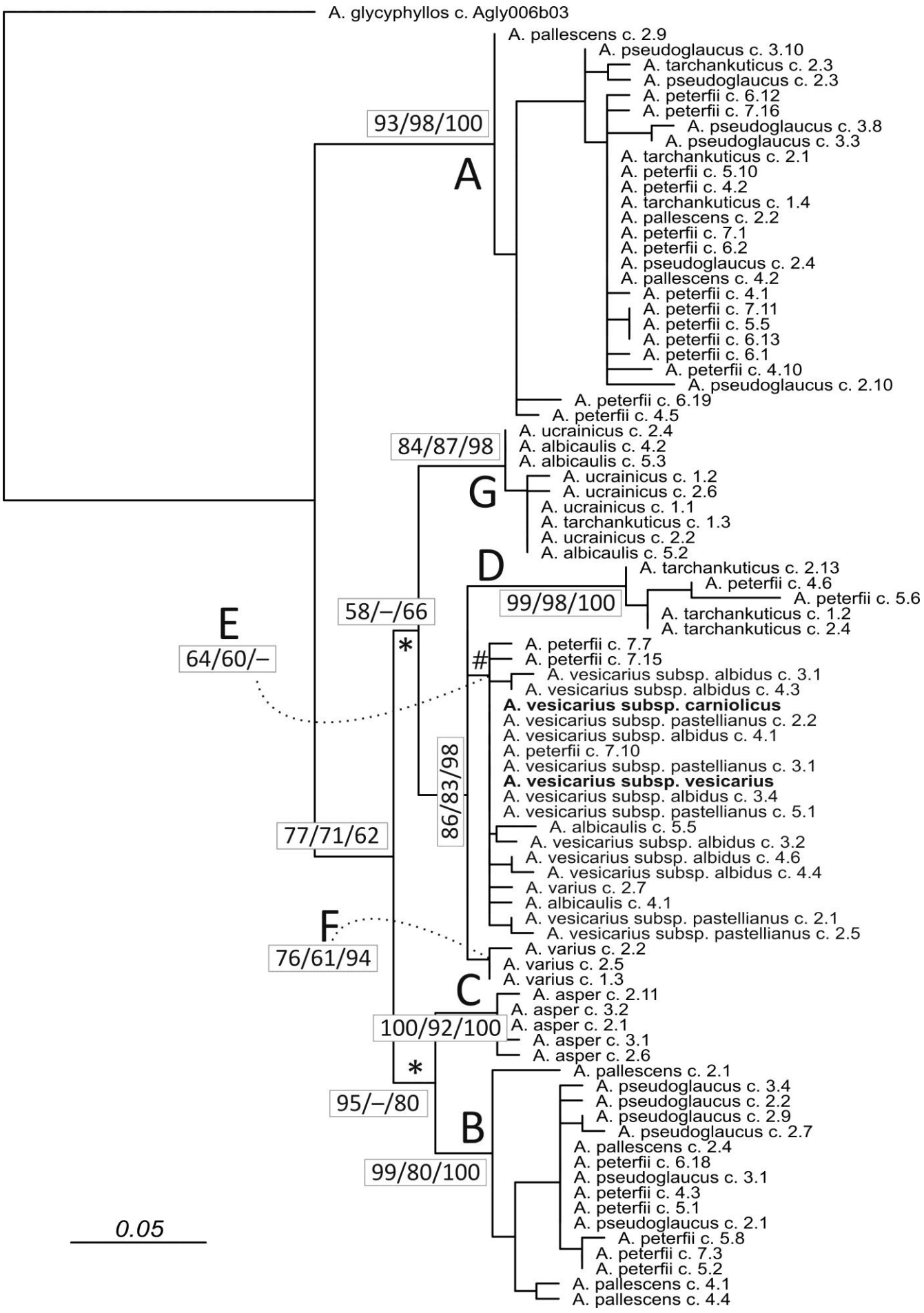
Fig. 1. Single most parsimonious tree resulted from the parsimony analysis of plastid dataset. Numbers adjacent to nodes represent maximum parsimony BS, Bayesian PP, and maximum likelihood BS percentages (not shown below 75%). The branch marked with asterisk collapses on the Bayesian majority rule consensus tree of the same dataset.

Fig. 2. TCS parsimony network of ribotypes occurring in the nrITS dataset (one cell from the network corresponds to one ribotype). Sequence names include unique identifiers: acronym, sample and clone numbers (see Table 1). The number in parenthesis after the name of several clones refers to the number of replicates identical with them within one accession. For the list of the identical nrITS copies per specimens consult the Appendix A1. The small hollow circles represent hypothetical (inferred) or not sampled ribotypes. The two epithets in bold refer to direct sequence of *A. vesicarius* subsp. *carniolicus* and *A. vesicarius* subsp. *vesicarius*, respectively.

Fig. 3. Best maximum likelihood phylogram for 84 (ingroup) *Astragalus* nrITS clone sequences and two (ingroup) nrITS direct sequences produced with RAxML under the GTR+G substitution model, using one *A. glycyphyllos* sequence as outgroup. Numbers adjacent to (relevant) nodes represent ML and MP bootstrap, as well as, Bayesian PP support percentages. Branches marked with asterisk collapse on the MP strict consensus tree of the same dataset. The branch marked with # collapses on the Bayesian majority rule consensus tree of the same dataset. Differences between the ML, MP and Bayesian trees below the level of clades A–G, as well as nodal support values for their subclades are not shown. The two direct sequences are in bold.


A. glycyphyllos
A. asper
A. ucrainicus
A. pseudoglaucus
76/100/–
A. vesicarius subs. pastellianus
90/100/97
A. albicaulis
A
98/100/96
A. tarchankuticus
A. pallescens
90/100/–
91/100/99
A. peterfii
A. varius
99/100/99
A. vesicarius subs. carniolicus
B
*
A. vesicarius subsp. vesicarius
87/100/89
A. vesicarius subsp. albidus


E
ves3.1(2)
alb4.1
pet7.7
pet7.15
ves3.2
alb5.5
ves4.3(2)
carniolicus
vesicarius
pas2.2(2)
pas3.1(5)
pas5.1(4)
pet7.10(2)
ves3.4(2)
ves4.1
ves4.6
var2.7
pas2.1(3)
ves4.4
pas2.5
F
var2.2
var1.3(4)
var2.5(6)
G
ucr1.2
alb5.2(2)
tar1.3
ucr1.1(8)
ucr2.2(2)
alb4.2(4)
alb5.3(3)
ucr2.4
ucr2.6
D
tar2.13
tar1.2(4)
tar2.4(4)
pet4.6
pet5.6
B
gla2.7
gla2.9
gla2.1(3)
gla3.1(5)
pal2.4
pet4.3
pet5.1(4)
pet6.18(2)
pet5.2
pet7.3
gla3.4
pet5.8
gla2.2
pal4.4
pal4.1(2)
pal2.1
B
C
asp2.1(5)
asp3.2(4)
asp2.11
asp2.6
asp3.1
A
gla2.10
gla3.8
gla3.3
pet4.10
gla2.4(2)
pal2.2(6)
pal4.2(3)
pet4.2(3)
pet5.10
pet6.2(8)
pet7.1(10)
tar1.4(4)
tar2.1(3)
pet4.1
pet6.1(7)
pet6.12
pet7.16
pal2.9
pet6.19
gla3.10
pet4.5
tar2.3(2)
gla2.3
pet5.5
pet6.13
pet7.11(2)


A. glycyphyllos c. Agly006b03
A. pallescens c. 2.9
A. pseudoglaucus c. 3.10
A. tarchankuticus c. 2.3
A. pseudoglaucus c. 2.3
A. peterfii c. 6.12
A. peterfii c. 7.16
A. pseudoglaucus c. 3.8
A. pseudoglaucus c. 3.3
A. tarchankuticus c. 2.1
A. peterfii c. 5.10
A. peterfii c. 4.2
A. tarchankuticus c. 1.4
A. pallescens c. 2.2
A. peterfii c. 7.1
A. peterfii c. 6.2
A. pseudoglaucus c. 2.4
A. pallescens c. 4.2
A. peterfii c. 4.1
A. peterfii c. 7.11
A. peterfii c. 5.5
A. peterfii c. 6.13
A. peterfii c. 6.1
A. peterfii c. 4.10
A. pseudoglaucus c. 2.10
A. peterfii c. 6.19
A. peterfii c. 4.5
A. ucrainicus c. 2.4
A. albicaulis c. 4.2
A. albicaulis c. 5.3
A. ucrainicus c. 1.2
A. ucrainicus c. 2.6
A. ucrainicus c. 1.1
A. tarchankuticus c. 1.3
A. ucrainicus c. 2.2
A. albicaulis c. 5.2
A. tarchankuticus c. 2.13
A. peterfii c. 4.6
A. peterfii c. 5.6
A. tarchankuticus c. 1.2
A. tarchankuticus c. 2.4
A. peterfii c. 7.7
A. peterfii c. 7.15
A. vesicarius subsp. albidus c. 3.1
A. vesicarius subsp. albidus c. 4.3
A. vesicarius subsp. carniolicus
A. vesicarius subsp. pastellianus c. 2.2
A. vesicarius subsp. albidus c. 4.1
A. peterfii c. 7.10
A. vesicarius subsp. pastellianus c. 3.1
A. vesicarius subsp. vesicarius
A. vesicarius subsp. albidus c. 3.4
A. vesicarius subsp. pastellianus c. 5.1
A. albicaulis c. 5.5
A. vesicarius subsp. albidus c. 3.2
A. vesicarius subsp. albidus c. 4.6
A. vesicarius subsp. albidus c. 4.4
A. varius c. 2.7
A. albicaulis c. 4.1
A. vesicarius subsp. pastellianus c. 2.1
A. vesicarius subsp. pastellianus c. 2.5
A. varius c. 2.2
A. varius c. 2.5
A. varius c. 1.3
A. asper c. 2.11
A. asper c. 3.2
A. asper c. 2.1
A. asper c. 3.1
A. asper c. 2.6
A. pallescens c. 2.1
A. pseudoglaucus c. 3.4
A. pseudoglaucus c. 2.2
A. pseudoglaucus c. 2.9
A. pseudoglaucus c. 2.7
A. pallescens c. 2.4
A. peterfii c. 6.18
A. pseudoglaucus c. 3.1
A. peterfii c. 4.3
A. peterfii c. 5.1
A. pseudoglaucus c. 2.1
A. peterfii c. 5.8
A. peterfii c. 7.3
A. peterfii c. 5.2
A. pallescens c. 4.1
A. pallescens c. 4.4
93/98/100
84/87/98
58/–/66
64/60/–
86/83/98
77/71/62
76/61/94
100/92/100
95/–/80
99/80/100
99/98/100
A
G
D
E
F
C
B
*
#
*
0.05

Supplementary Table S1. Variable nucleotide positions in the alignment of 54 unique ribotypes retrieved from the nrITS dataset. Ribotypes are sorted according to the genetically similar groupings (A–G) they belong to. Dots indicate the presence of the same nucleotide of first sequence in the same column, whereas hyphen indicates gap.

		nucleotide position	6	18	30	34	39	51	58	67	89	90	91	92	93	94	118	121	169	173	181	191	194	196	218	241	291	304	317	342	355
ribotype groups	A	gla2.4	G	G	A	T	G	-	A	C	G	T	G	T	G	A	T	C	T	C	C	A	T	T	G	G	G	G	C	G	C
		pet5.5	.	A	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		pet4.1	.	.	G	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		pet6.1	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		pet6.12	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		pet7.16	.	.	.	.	.	-	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		pet4.10	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		gla2.10	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		gla3.8	.	.	.	.	.	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		gla3.3	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		gla3.10	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		gla2.3	.	.	.	.	C	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		tar2.3	.	.	.	.	C	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
		pal2.9	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
		pet4.5	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
		pet6.19	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
	B	gla2.1	.	.	.	.	.	-	C	.	.	C	C	G	C	.	G	.	C	.	T	T	.	.	.	.	.	.	.	.	.	A
		gla2.9	.	.	.	.	.	-	C	.	.	C	C	G	C	.	G	.	C	.	T	T	G	.	.	.	.	.	.	.	A
		gla2.7	.	.	.	.	.	-	C	A	.	C	C	G	C	.	G	.	C	.	T	T	G	.	.	.	.	.	.	.	A
		gla3.4	.	.	.	.	.	-	C	.	.	C	C	G	C	.	G	.	C	.	T	T	.	.	.	.	.	.	.	.	.	A
		gla2.2	.	.	.	.	.	-	C	.	.	C	C	G	C	G	G	.	C	.	T	T	.	.	.	.	.	.	.	.	.	A
		pet5.2	.	.	.	C	.	-	C	.	.	C	C	G	C	.	G	.	C	.	T	T	.	.	.	.	.	.	.	.	.	A
		pet5.8	.	.	.	C	.	-	C	.	.	C	C	G	C	.	G	.	C	.	T	T	.	.	.	.	.	.	.	.	.	A
		pal4.4	.	.	.	.	.	-	C	T	.	C	C	G	C	.	G	.	C	.	.	T	.	.	.	.	.	.	.	.	.	A
		pal4.1	T	.	.	.	.	-	C	T	.	C	C	G	C	.	G	.	C	.	.	T	.	.	.	.	.	.	.	.	.	A
		pal2.1	A	.	.	.	.	-	C	T	.	C	C	G	C	.	G	.	C	.	.	T	.	.	.	.	.	A	.	T	A

Supplementary Table S1. (continuation)

ribotype groups			356	369	386	397	402	415	416	421	426	430	431	435	450	453	472	480	486	491	492	502	503	506	507	549	561	564	567	571	578	589
	A	gla2.4	G	C	T	G	C	A	T	G	T	G	T	A	A	G	C	C	C	T	G	A	T	T	-	T	G	C	T	A	C	G
		pet5.5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet4.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet6.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet6.12	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet7.16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet4.10	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	.	.
		gla2.10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		gla3.8	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		gla3.3	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		gla3.10	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		gla2.3	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		tar2.3	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pal2.9	.	.	.	.	.	.	.	A	.	.	C	T	.	.	.	.	.	.	A	.	.	.	-	.	.	.	.	.	.	.
		pet4.5	.	.	.	.	.	.	.	A	.	.	C	T	.	A	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.
		pet6.19	.	.	.	.	.	.	.	A	.	.	C	T	.	.	.	.	.	.	.	.	.	.	-	.	A	.	.	G	.	.
	B	gla2.1	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		gla2.9	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		gla2.7	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		gla3.4	.	T	.	.	.	G	.	A	.	.	C	T	T	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		gla2.2	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		pet5.2	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	T	.	.	.	.
		pet5.8	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	.	.	.	.	.
		pal4.4	.	T	.	.	.	G	.	.	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	.	.	.	.	.
		pal4.1	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	G	A	.	.	.	-	.	.	.	.	.	.	.
		pal2.1	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	.	.	A	.	.	.	-	.	.	T	.	.	.	.

Supplementary Table S1. (continuation)

ribotype groups			6	18	30	34	39	51	58	67	89	90	91	92	93	94	118	121	169	173	181	191	194	196	218	241	291	304	317	342	355
	(A)	gla2.4	G	G	A	T	G	-	A	C	G	T	G	T	G	A	T	C	T	C	C	A	T	T	G	G	G	G	C	G	C
	C	asp2.1	.	.	.	.	.	G	C	T	T	.	T	G	.	.	G	.	C	.	.	T	.	.	.	.	.	.	.	.	.	A
		asp2.11	.	.	.	.	.	G	C	T	T	.	T	G	.	.	G	.	C	T	.	T	.	.	.	.	.	.	.	.	.	A
		asp2.6	.	.	.	.	.	G	C	T	T	.	T	G	.	.	G	.	C	.	.	T	.	.	.	.	.	.	.	.	.	A
		asp3.1	.	.	.	.	.	G	C	T	T	.	T	G	.	.	G	.	C	.	.	T	.	G	.	.	.	.	.	.	.	A
	D	tar1.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	.	.	T	.	.	.	T	T	.	T	A	A
		tar2.13	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	.	.	T	.	.	.	.	T	.	T	A	A
		pet4.6	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	.	.	T	.	.	.	T	T	.	T	A	A
		pet5.6	.	.	G	.	.	-	C	T	.	.	A	G	.	.	G	.	C	.	.	T	.	.	.	T	T	.	T	A	A
	E	pas.2.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		var2.7	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	G	.	T	.	.	.	.	.	.	.	.	A
		ves4.3	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		ves3.1	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		alb4.1	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	C	.	.	.	.	.	.	.	A
		pet7.7	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		pet7.15	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	A	T	.	.	.	.	.	.	.	.	A
		ves3.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		alb5.5	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		ves4.6	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		ves4.4	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		pas2.1	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		pas2.5	.	.	.	.	.	-	C	T	.	.	A	G	.	.	A	.	C	.	.	T	.	.	.	.	.	.	.	.	A
	F	var1.3	.	.	.	.	.	-	C	T	.	.	A	G	.	C	G	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		var2.5	.	.	.	.	.	-	C	T	.	.	A	G	.	C	G	.	C	.	.	T	.	.	.	.	.	.	.	.	A
		var2.2	.	.	.	.	.	-	C	T	.	.	A	G	.	C	G	.	C	.	.	T	.	.	.	.	.	.	.	.	A
	G	alb5.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	A	.	T	.	C	T	.	.	.	.	.	A
		alb4.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	A	.	T	.	C	T	.	.	.	.	.	A
		ucr1.2	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	A	.	T	.	C	T	.	.	.	.	.	A
		ucr2.6	.	.	.	.	.	-	C	T	.	.	A	G	.	.	G	.	C	A	.	T	.	C	T	.	.	.	.	.	A

Supplementary Table S1. (continuation)

ribotype groups			356	369	386	397	402	415	416	421	426	430	431	435	450	453	472	480	486	491	492	502	503	506	507	549	561	564	567	571	578	589
	(A)	gla2.4	G	C	T	G	C	A	T	G	T	G	T	A	A	G	C	C	C	T	G	A	T	T	-	T	G	C	T	A	C	G
	C	asp2.1	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	A	.	A	.	.	.	-	-	.	T	.	.	.	.
		asp2.11	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	A	.	A	.	.	.	-	-	.	T	.	.	.	.
		asp2.6	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	A	.	A	.	C	.	-	-	.	T	.	.	.	.
		asp3.1	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	T	A	.	A	.	.	.	-	-	.	T	.	.	.	.
	D	tar1.2	A	T	.	.	A	G	C	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	A	T	.	.	.	A
		tar2.13	A	T	.	.	A	G	C	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	A	T	.	.	.	A
		pet4.6	A	T	.	.	A	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	A	-	.	A	T	.	.	.	A
		pet5.6	A	T	.	.	A	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	A	-	.	.	.	.	.	.	.
	E	pas2.2	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		var2.7	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		ves4.3	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	G	.	.	-	.	.	T	.	.	.	A
		ves3.1	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	T	.	.	-	.	.	T	.	.	.	A
		alb4.1	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		pet7.7	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	C	A	.	.	.	-	.	.	T	.	.	.	A
		pet7.15	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		ves3.2	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	T	.	A
		alb5.5	.	T	C	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	G	.	A
		ves4.6	.	T	A	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		ves4.4	.	T	A	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	C	.	-	.	.	T	.	.	.	A
		pas2.1	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	C	.	-	.	.	T	.	.	.	A
		pas2.5	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	C	.	-	.	.	T	.	.	T	A
	F	var1.3	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	-	.	.	T	.	.	.	A
		var2.5	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	T	.	.	T	.	.	.	A
		var2.2	.	T	.	.	.	G	.	A	.	.	C	T	G	T	T	.	.	.	A	.	.	.	T	.	.	T	C	.	.	A
	G	alb5.2	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	.	.	.	A	.	.	.	-	.	.	.	C	.	.	A
		alb4.2	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	.	.	.	A	.	.	.	-	.	.	.	.	.	.	A
		ucr1.2	.	T	.	.	.	G	.	A	.	.	C	T	.	.	T	.	.	.	A	.	.	.	-	.	.	T	C	.	.	A
		ucr2.6	.	T	.	.	.	G	.	A	.	.	.	T	.	.	T	.	.	.	A	.	.	.	-	.	.	.	C	.	.	A

Appendix A1. Distribution of nrITS clone sequences among the ribotype groups identified in the parsimony network analysis. The nrITS clone sequences within a given ribotype group have their unique identifiers: acronym of species and number of individual (see Table 2), number of clone sequence. Identical sequences from the same accession are in the same row and share the same GenBank accession number.

A	gla2.10	HQ241855
	gla2.3	HQ241858
	gla2.4, gla2.6	HQ241841
	gla3.10	HQ241859
	gla3.3	HQ241856
	gla3.8	HQ241857
	pal2.2, pal2.3, pal2.5, pal2.6, pal2.7, pal2.8	HQ241842
	pal2.9	HQ241860
	pal4.2, pal4.8, pal4.9	HQ241843
	pet4.1	HQ241861
	pet4.10	HQ241863
	pet4.2, pet4.7, pet4.8	HQ241844
	pet4.5	HQ241862
	pet5.10	HQ241845
	pet5.5	HQ241851
	pet6.1, pet6.3, pet6.5, pet6.7, pet6.8, pet6.21, pet6.23	HQ241850
	pet6.12	HQ241864
	pet6.13	HQ241852
	pet6.19	HQ241865
	pet6.2, pet6.4, pet6.6, pet6.9, pet6.11, pet6.15, pet6.16, pet6.17	HQ241846
	pet7.1, pet7.2, pet7.4, pet7.5, pet7.8, pet7.13, pet7.14, pet7.18, pet7.19, pet7.21	HQ241847
	pet7.11, pet7.20	HQ241853
	pet7.16	HQ241866
	tar1.4, tar1.7, tar1.12, tar1.13	HQ241848
	tar2.1, tar2.2, tar2.5	HQ241849
	tar2.3, tar2.6	HQ241854
B	gla2.1, gla2.5, gla2.8	HQ241867
	gla2.2	HQ241876
	gla2.7	HQ241877
	gla2.9	HQ241878
	gla3.1, gla3.2, gla3.5, gla3.6, gla3.7	HQ241868
	gla3.4	HQ241879
	pal2.1	HQ241880
	pal2.4	HQ241869
	pal4.1, pal4.3	HQ241873
	pal4.4	HQ241881
	pet4.3	HQ241870
	pet5.1, pet5.4, pet5.7, pet5.9	HQ241871
	pet5.2	HQ241874
	pet5.8	HQ241882
	pet6.18, pet6.22	HQ241872
	pet7.3	HQ241875
C	asp2.1, asp2.3, asp2.8, asp2.9, asp2.10	HQ241883
	asp2.11	HQ241886
	asp2.6	HQ241885
	asp3.1	HQ241887
	asp3.2, asp3.4, asp3.5, asp3.6	HQ241884
D	pet4.6	HQ241890
	pet5.6	HQ241891
	tar1.2, tar1.5, tar1.6, tar1.9	HQ241888
	tar2.13	HQ241892
	tar2.4, tar2.7, tar2.10, tar2.12	HQ241889

E	alb4.1	HQ241901
	alb5.5	HQ241902
	pas2.1, pas2.3, pas2.6	HQ241898
	pas2.2, pas2.4	HQ241893
	pas2.5	HQ241903
	pas3.1, pas3.2, pas3.3, pas3.4, pas3.5	HQ241894
	pas5.1, pas5.3, pas5.4, pas5.6	HQ241895
	pet7.10, pet7.12	HQ241896
	pet7.15	HQ241904
	pet7.7	HQ241905
	var2.7	HQ241906
	ves3.1, ves3.3	HQ241899
	ves3.2	HQ241907
	ves3.4, ves3.5	HQ241897
	ves4.1	HQ241908
	ves4.3, ves4.5	HQ241900
	ves4.4	HQ241909
	ves4.6	HQ241910
F	var1.3, var1.4, var1.5, var1.6	HQ241912
	var2.2	HQ241913
	var2.5, var2.6, var2.8, var2.9, var2.11, var2.14	HQ241911
G	alb4.2, alb4.3, alb4.4, alb4.5	HQ241918
	alb5.3, alb5.8, alb5.10	HQ241919
	alb5.2, alb5.12	HQ241914
	tar1.3	HQ241915
	ucr1.1, ucr1.3, ucr1.4, ucr1.5, ucr1.8, ucr1.9, ucr1.10, ucr1.11	HQ241916
	ucr1.2	HQ241921
	ucr2.2, ucr2.3	HQ241917
	ucr2.4	HQ241920
	ucr2.6	HQ241922