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Organization of Chloroplast *psb*A-*trn*H Intergenic Spacer in Dicotyledonous Angiosperms of the Family Umbelliferae

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Abstract—Chloroplast intergenic *psb*A-*trn*H spacer has recently become a popular tool in plant molecular phylogenetic studies at low taxonomic level and as suitable for DNA barcoding studies. In present work, we studied the organization of *psb*A-*trn*H in the large family Umbelliferae and its potential as a DNA barcode and phylogenetic marker in this family. Organization of the spacer in Umbelliferae is consistent with a general pattern evident for angiosperms. The 5′-region of the spacer situated directly after the *psb*A gene is more conserved in length compared to the 3′-region, which has greater sequence variation. This pattern can be attributed to the maintenance of the secondary structural elements in the 5′-region of the spacer needed for posttranscriptional regulation of *psb*A gene expression. In Umbelliferae only, the conserved region contains a duplication of the fragment corresponding to the loop of the stem–loop structure and an independent appearance of identical sequence complementarities (traits) necessary to stabilize the stem–loop structure in different lineages. The 3′-region of the spacer nearest to *trn*H ranges greatly in size, mainly due to deletions, and the decrease in spacer length is a general trend in the evolution *psb*A-*trn*H in Umbelliferae. The features revealed in spacer organization allow us to use it as phylogenetic marker, and indels seem to be more informative for analyses than nucleotide substitutions. However, high conservation among closely related taxa and occurrence of homoplastic inversions in the stem–loop structure limit its application as DNA barcode.

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Key words: *psb*A-*trn*H, secondary structure, phylogeny, DNA barcoding, Umbelliferae

Analysis of nucleotide sequences has become an integral part of the study of the evolutionary history of organisms. The main objective in this case is to choose a sequence where the frequency of occurrence of substitutions is consistent with the stated taxonomic problem [1]. Selecting regions of DNA and analyzing their structure, we can compare the partial structure of the genotypes of plants of any degree of phylogenetic relationship [2]. The chloroplast genome provides a unique opportunity due to its multicopy and intragenomic homogeneity. Introns of genes and intergenic spacers, which are rapidly evolving noncoding sequences, are used to study the relationship on a low taxonomic level. The widespread use of nucleotide sequences in the study of relationships between organisms has led to the development of the approach of DNA barcoding, where some DNA regions are used to identify species [3-5]. The analysis of datasets, which have included a number of representatives of both monocots and dicots, have shown that the chloroplast intergenic spacer *psb*A-*trn*H is most successfully amplified using universal primers, and it has the optimum ratio of length and number of informative positions [6] when compared with other noncoding sequences of the genome. This spacer is located between the *psb*A gene and the gene of histidine transfer RNA (*trn*H) and plays an important role in the regulation of expression of these genes. The gene *psb*A encodes protein D1, which together with protein D2 comprises the reaction center of photosystem II. In most angiosperms, including carrot (*Daucus carota*) of the family Umbelliferae, this gene is localized in the large single copy region. The usefulness of the *psb*A-*trn*H spacer as a phylogenetic marker at lower taxonomic levels has been demonstrated in the studies of such genera as

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Rhododendron [7], *Clematis* [8], *Petunia* [9], *Dendrobium* [10], and *Compsoneura* [11].

Umbelliferae is one of the largest and agriculturally important families of the angiosperms. Many Umbelliferae are highly regarded as essential oil plants (coriander, cumin), vegetables (carrots, parsley, celery, fennel), and drug plants (dill, ferule). The main difficulty in the systematics of the Umbelliferae family is associated with isolation of supraspecific taxa. Multidisciplinary research on the Umbelliferae has been done for many years at Moscow State University, where comparative analysis of DNA sequences has made an indispensable contribution. A molecular classification of the family, based mainly on sequences of internal transcribed spacers (ITS) of nuclear ribosomal DNA [12, 13], was developed during the past decade. However, the results are often at odds with existing systems based on morphological characters. Therefore, to test the phylogenetic hypotheses obtained by analyzing the ITS region of nuclear DNA, we used the *psb*A-*trn*H spacer of the chloroplast DNA. This allowed us not only to obtain important information about the relationship of taxa, but also to trace possible changes in the organization of the *psb*A-*trn*H spacer in a variety of related groups and to assess its variability at different taxonomic levels and for the usability in molecular phylogenetic analysis and species identification.

METHODS OF INVESTIGATION

Taxa were selected in accordance with available data on the taxonomy and phylogeny of the Umbelliferae and includes representatives of the three major Umbelliferae subfamilies: Apioideae (63 genera, 231 species), Saniculoideae (3 genera, 5 species), and Mackinlayoideae (1 genus, 1 species). The set includes also representatives of the family Araliaceae (4 genera, 6 species) – the closest to Umbelliferae group of plants. We selected sequences (223) that we have previously reported [14-16] and also data of other authors (25) that is deposited in the GenBank database (www.ncbi.nlm.nih.gov). A total of 248 sequences were analyzed (see Appendix).

The nucleotide sequences were aligned automatically using the Muscle software [17] and were manually edited using BioEdit [18]. The DotHelix program [19] was used for detection of the boundaries of the indels in the alignment. To determine the positional homology of nucleotides disrupted by inversions modeling of the secondary structure with the use of interactive program RNA mfold with default settings (http://mfold.rna.albany.edu) was performed [20]. Secondary structure was represented graphically using RNA-viz 2.0 [21]. During the secondary structure modeling, DNA sequences were treated as RNA transcripts. The level of divergence was assessed by *p*-distances.

RESULTS

Both in Umbelliferae and in Araliaceae the *psb*A-*trn*H spacer sequences vary greatly in length (Fig. 1). Spacer length varies from 248 to 405 bp in Araliaceae and from 110 to 417 bp in the Umbelliferae. Such differences in the length of the spacer in Araliaceae and Umbelliferae are mainly related to the presence of a large number of deletions. Thus, depending on the length of the spacer and distribution of its indels, taxa can be divided into groups corresponding to the classification based on ITS sequences [13].

Despite significant differences in length, some parts of the *psb*A-*trn*H spacer are highly conserved in the Umbelliferae family and between the families Araliaceae and Umbelliferae (Fig. 2). The first conserved site (C1) is located directly behind the stop codon of gene *psb*A (TAA). It has 41-63 bp length. Slight differences in the length of the spacer are associated with a difference in the length of homopolymeric stretches formed by A or T nucleotides, as well as duplications or short indels (2-6 bp). Independent occurrences of similar substitutions (TCT-motif) in poly(A)-stretch in species of the genera *Bunium*, *Elaeosticta*, and *Schrenkia* from group 1.1 of the tribe Pyramidoptereae were found. A TT-motif was found in tribe Scandiceae and Hymenidium-II group.

After the C1 region there is a variable region (V1), which varies both in length and in nucleotide composition: it is composed predominantly of purine bases in some species and of the pyrimidine in some other species. The observed differences are due to small inversions flanked by short inverted repeats. Analysis of secondary structure showed that a significant portion of the V1 region is involved in the formation of a helix by inverted repeats. Bases that form the polymorphic region are not involved in the complementary interaction, and they form a hairpin loop. Thus, changes in the primary structure of the spacer do not affect or have a little effect on the stability of its secondary structure. We found that the hairpin stem has length of 19 to 41 bp. A duplication was observed in the 11-nucleotide fragment that corresponds to the hairpin loop in genera *Heracleum* (cow parsnip), *Pastinaca* (parsnip), and *Leiotulus* belonging to group 5.1 of tribe Tordylieae. An inversion of both the sequence corresponding to the hairpin loop and duplicated fragment was also observed in this group (Fig. 3).

The conserved region C2 following the variable region V1 is a fragment of the inverted repeat complementary to a fragment of the region C1 (Fig. 2). Variable V2 region is characterized by the highest variations in length (17-327 bp), and indels observed in *psb*A-*trn*H spacer are localized mainly in this region. In group 1.1 of tribe Pyramidoptereae this region is almost completely lost. Depending on the length of the region and distribution of the deletions, all taxa can be divided into groups

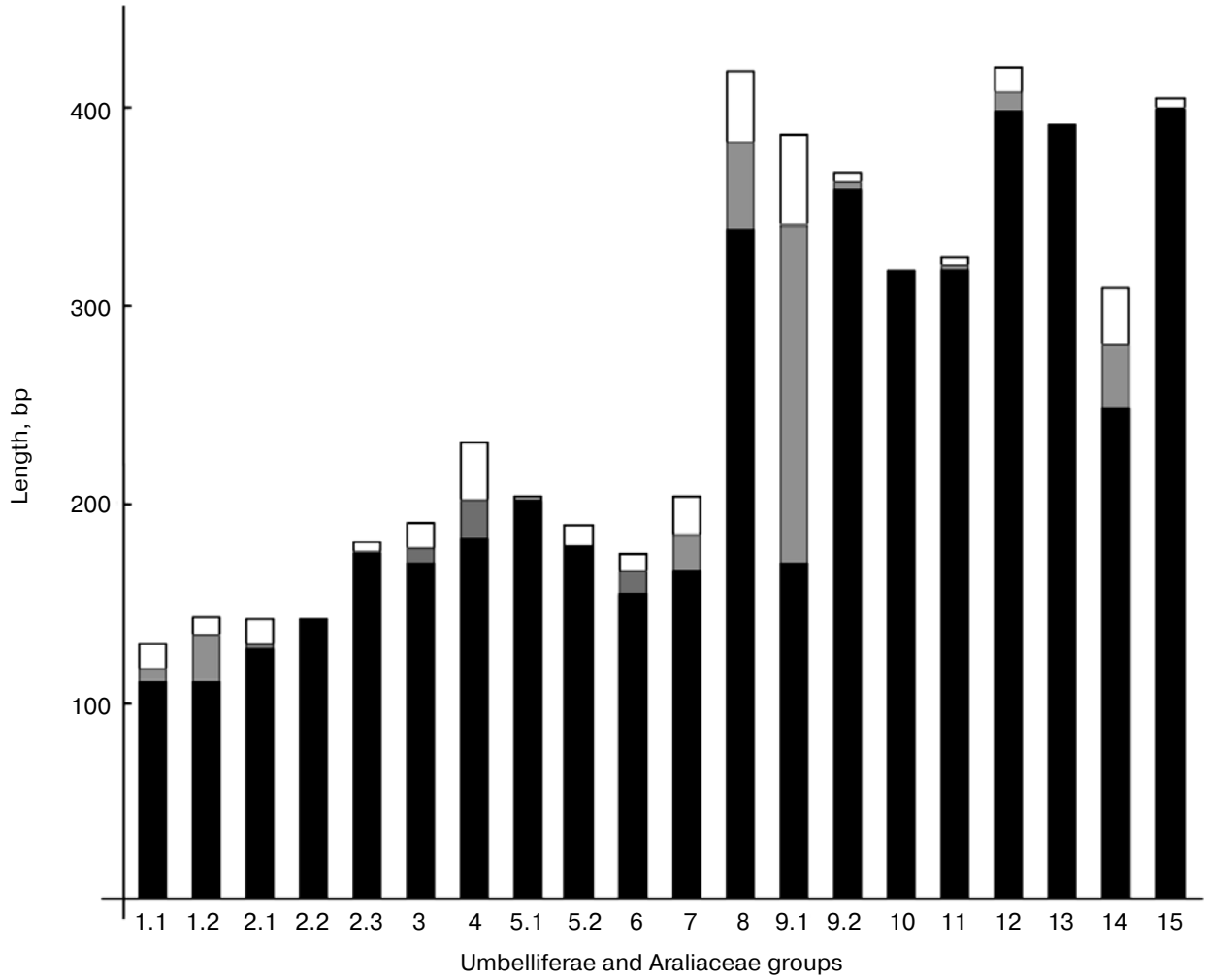

Fig. 1. Differences in length observed in the *psb*A-*trn*H spacer. Main Umbelliferae groups correspond to clades revealed in the analysis of ITS sequences of nuclear ribosomal DNA according to the classification based on molecular data [13]. Black shows the minimum value, gray shows the median value, and white shows the maximum value. Groups: 1, tribe Pyramidoptereae; 2, tribe Careae; 3, tribe Coriandreae; 4, tribe Selineae; 5, tribe Tordylieae; 6, tribe Scandiceae; 7, group of species Hymenidium-III; 8, clade Physospermopsis; 9, tribe Pleurospermeae; 10, tribe Bupleureae; 11, tribe Heteromorpheae. Groups 1-11 are a subfamily of Apioideae. Group 12, subfamily Saniculoideae. Group 13, subfamily Mackinlayoideae. Groups 14 and 15 correspond to the family Araliaceae.

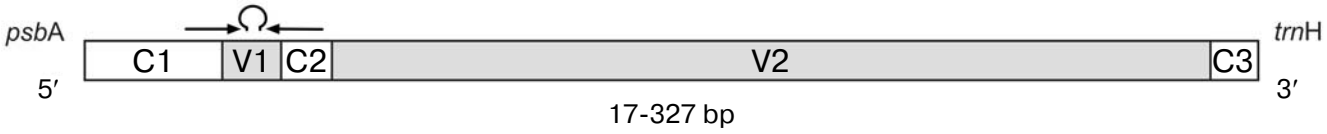

Fig. 2. General scheme of organization of the *psb*A-*trn*H spacer. Conserved (C1, C2, C3) and variable (V1, V2) regions are marked.

corresponding to the classification based on sequences of ITS (Fig. 4). The conserved C3 region is presented by 4 or 5 nucleotides directly adjacent to the *trn*H gene.

In all the studied taxa the *psb*A-*trn*H spacer sequence is characterized by a high content of AT-bases (69-77%), which is typical for noncoding regions of chloroplast genome of plants in general and for this spacer in particular.

To understand the possible intraspecific variation in *psb*A-*trn*H, nucleotide sequences of 20 samples of *Heracleum sibiricum* (which is a polymorphous and widely distributed species) from different parts of its distribution area were analyzed. It is shown that the spacer sequences in samples taken from different populations differ, and the differences are confined to region V1 and are due to the presence of inversions.

DISCUSSION

General characteristics of organization of the *psb*A-*trn*H spacer. The changes observed in the *psb*A-*trn*H spacer in the Umbelliferae and Araliaceae are consistent with the general scheme proposed for angiosperms [22]. Thus, the 5′-region of the spacer, which covers the C1, V1, and C2 regions in Fig. 2, is rather conserved in length (Fig. 3) and contains inverted repeats that form a hairpin, in contrast to the 3′-region (mainly V2 region). In bacteria, these hairpins act as a transcriptional terminator [23]. However, the inverted repeats in chloroplasts play another role – they do not serve as transcription terminators, but they play an important role in processing and stabilization of mRNA [24, 25]. The role of terminators in such cases could be attributed to tRNA. This assumption applies to the *psb*A-*trn*H spacer. It is believed that the *trn*H gene has no a functional promoter and is possibly co-transcribed with gene *psb*A [24, 26, 27]. Excision of tRNA from the primary transcript occurs with the participation of RNase P, which recognizes the characteristic folded structure of tRNA and does not require specific recognition sites [28].

The importance of the secondary structure of the 5′-region of spacers, which is the 3′-noncoding region of chloroplast genes, has been shown experimentally for some genes. Thus, the removal of the 3′-untranslated region of the *atp*B gene in *Chlamydomonas* reduces the amount of mRNA and the product of this gene [29]. Experimental data for the 3′-untranslated region of the *psb*A gene reveal a complex picture. On one hand, there is evidence that the secondary structure is necessary for the stabilization of the corresponding mRNA [24]. On the other hand, it was found that removal of part of the sequence that forms a helix has no such effect on mRNA stability, and some substitutions, on the contrary, increase it [30].

Interestingly, the region located between the inverted repeats that we identified as polymorphic (not only at interspecific but also intraspecific level) exhibits strictly channeled polymorphism. There are only two main variants of the structure of this region resulting from structural changes – inversions. Point substitutions in this region are rare. Perhaps this is connected with the fact that the structure of the hairpin loop is also important for maintaining the stability of the mRNA. One can also assume that this reflects a general trend in the evolution of chloroplast noncoding DNA – the prevalence of microstructural changes compared to point substitutions.

As in most angiosperms, in C1 region in the Umbelliferae and Araliaceae there is a short conserved motif that corresponds to the expected regulatory region, apparently responsible for the stability of the mRNA transcript and processing [22].

Peculiarities in organization of the *psb*A-*trn*H spacer in Umbelliferae. It was found that in the studied species of

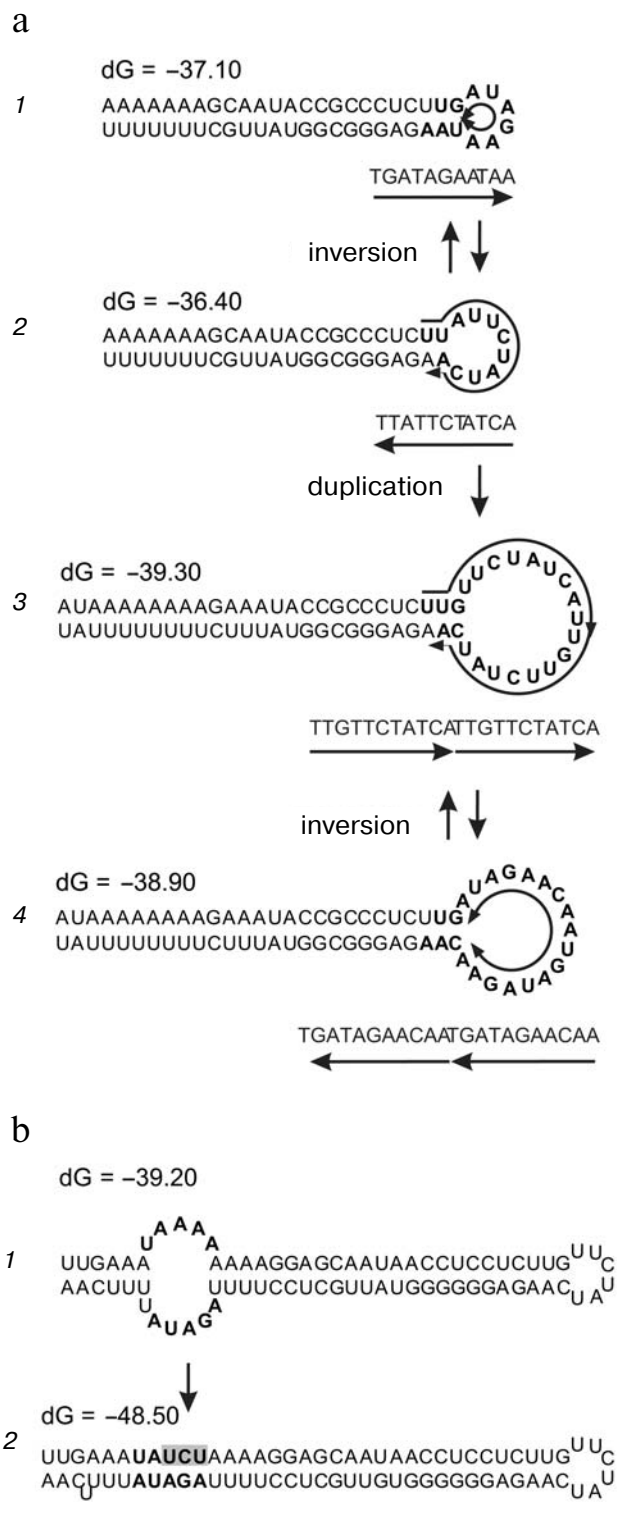

Fig. 3. Secondary structure of the spacer 5′-region that contains the hairpin. a) Duplication of the fragment corresponding to the hairpin loop that is observed in tribe Tordylieae (group 5.1): *1*) *Pastinacopsis glacialis*; *2*) *Semenovia heterodonta*; *3*) *Heracleum ponticum*; *4*) *Heracleum sibiricum*. b) Impact of changes on the hairpin stability that is observed in tribe Pyramidopteraе (group 1.1): *1*) *Bunium cylindricum*; *2*) *Bunium badachschanicum*.

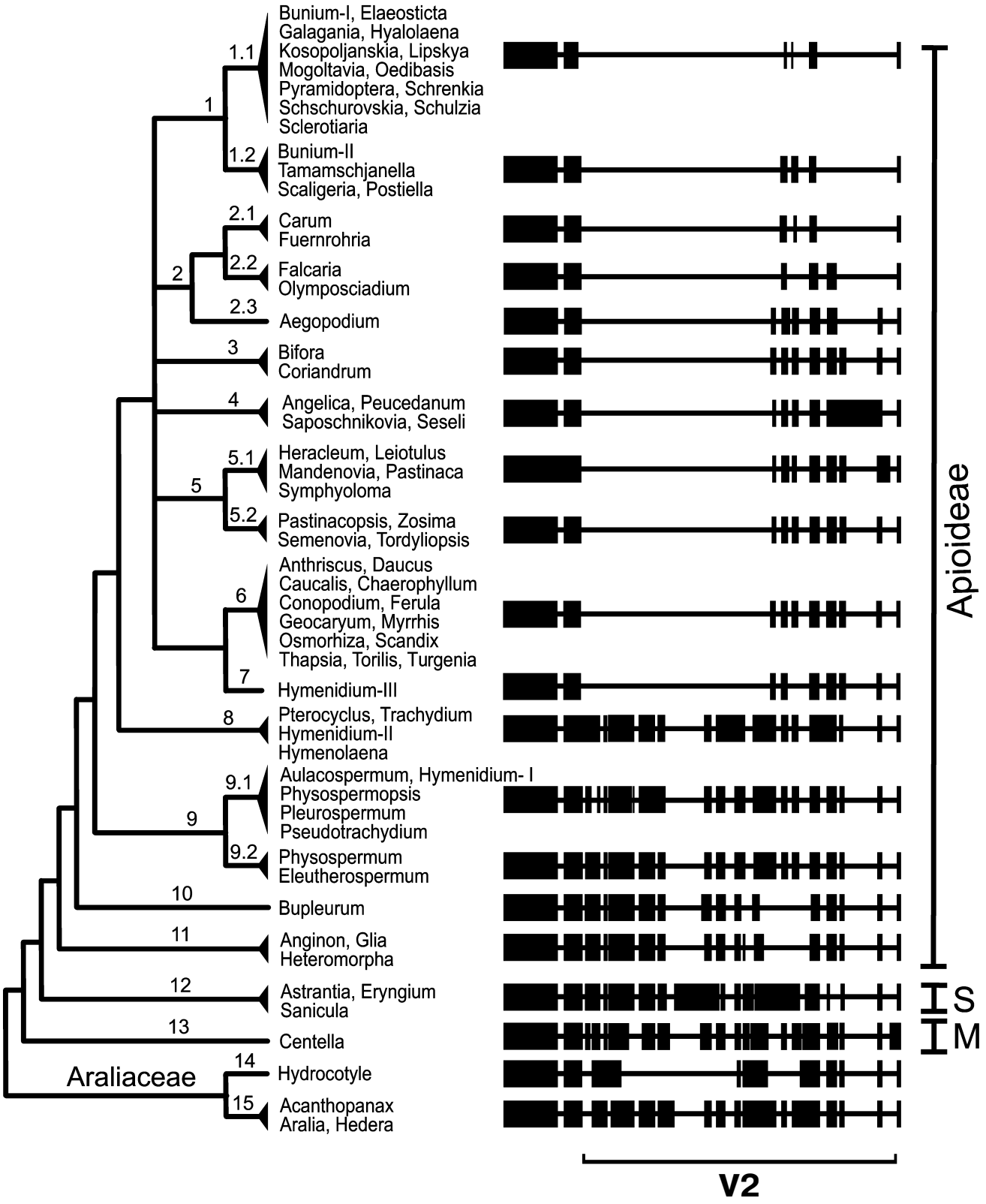

Fig. 4. Umbelliferae taxa relationships revealed by analysis of ITS sequences of a nuclear ribosomal spacer and indels observed in the *psb*A-*trn*H region marking individual clades. Abbreviations: S, subfamily Saniculoideae; M, subfamily Mackinlayoideae.

the Umbelliferae spacer sequences vary greatly in length. There is a tendency to loose certain fragments, mainly located in the 3′-region of the spacer, in a series of Araliaceae – basal Umbelliferae (Saniculoideae, Mackinlayoideae) – typical Umbelliferae (Apioideae). This trend is the greatest in group 1.1 of the tribe Pyramidoptereae, where the length of *psb*A-*trn*H is 110-129 bp. Most members of the subfamily Apioideae have spacer length of 178-200 bp. On the other hand, an increase in length that is apparently the result of amplifications of individual sites was observed within typical Umbelliferae in the tribe Selineae. Thus, the length of the spacer in this group reaches 230 bp, because it includes an AT-rich site of 32-71 bp in length, which showed no similarity to any sites of taxa from other groups. This feature is not typical for any of the other studied species, and therefore it is likely that the insertion of this site occurred in this group rather than its loss in all other species. This diversity of structure is probably due to the fact that this part does not have functional load.

Possibilities of using *psb*A-*trn*H spacer as a phylogenetic marker. It was found that in the sequences of the *psb*A-*trn*H spacer being analyzed in Umbelliferae replacements in the 5′-region (regions C1 and V1) can be homoplastic. First of all, it is the presence of inversions between inverted repeats (region V1). Similar inversions were observed among different genera and also within the same species (*Heracleum sibiricum*). As shown in several studies by other researchers [31-34], an inversion in such structures occurs very often and does not correlate with phylogeny. Our data suggest that these patterns are typical for the *psb*A-*trn*H region. Therefore, during the molecular-phylogenetic analysis, the fragment of the spacer that is located between the inverted repeats should be excluded.

In addition, in the conserved region C1 in group 1.1 of the tribe Pyramidoptereae characterized by extremely short spacer, similar replacements were found in taxa that are not grouped together in the analysis of ITS sequences and strongly separated morphologically. Thus, similar replacements (TCT motif) were observed in some species within the genera *Bunium*, *Elaeosticta*, and *Schrenkia*. In some cases the presence of such changes is the only significant difference between the compared taxa. Analysis of secondary structures of 5′-region of the spacer showed that such nucleotide substitutions contribute to the formation of hairpins having a more extended stem. We believe that such substitutions contribute to the stabilization of the secondary structure of the 5′-region of spacer. Therefore, substitutions observed in conserved regions must be used with caution, considering the possible major restructuring during the evolution of the spacer.

Elongation of the stem of the hairpin in the 5′-region of the spacer via nucleotide substitutions and complementary pairing of regions adjacent to inverted repeats was observed for different groups of angiosperms [22]. However, in the Umbelliferae family the independent occurrence of similar replacements for stabilization of the secondary structure of the hairpin in various related lines was first identified.

It is interesting to note that in the group comprising representatives of the tribe Scandiceae and a group of Hymenidium-II species, the appearance of specific replacements (TT-motif) was also observed in a poly(A)-stretch. However, this motif is not included in the hairpin stem and will not affect its stability.

The most interesting for studying phylogeny are microstructural changes (insertion/deletion) rather than nucleotide substitutions in the sequences of *psb*A-*trn*H (which are, first, few in number, and, second, have high level of homoplasia) (Fig. 4). In particular, the unique insertion of 10 nucleotides in *Heracleum* species and related genera distinguishes them from the *Semenovia* species, which are sometimes combined with them in classifications based on morphology.

Nevertheless, the *psb*A-*trn*H spacer can be used in molecular-phylogenetic analysis, taking into consideration that maximal attention should be given to insertions/deletions of individual fragments rather than single nucleotide substitutions.

Possible application of *psb*A-*trn*H spacer for DNA barcoding. Elucidation of relationships of taxa of Umbelliferae and facilitation of their identification is important for the systematics of the family, as well as for practical applications such as analysis of medicinal plants (there are reported cases of side effects for drugs, and the possibility cannot be excluded that another poisonous species was taken for production by mistake or through ignorance). DNA sequences selected for barcoding should be: 1) highly variable (vary at the species level); 2) short (no more than 700-800 bp) to facilitate selection, amplification, and sequencing, and 3) be flanked with conserved sites, so work can be done with universal primers, and in the future make it easier to align the sequences. Taking into account these requirements, a *psb*A-*trn*H spacer has been proposed as one of the plant barcode [6].

To evaluate the possibility of using a *psb*A-*trn*H spacer as a DNA barcode, we analyzed the degree of variability at different taxonomic levels. Thus, the level of intraspecific variation (exemplified by 20 samples of *Heracleum sibiricum*) is determined by the presence or absence of inversions, which are confined to the site located between two inverted repeats, and are not due to nucleotide substitutions but inversion. These inversions show no correlation with geographic distribution and appear to have occurred independently in different populations.

The level of intrageneric differences amounted to 2.6% (*Schrenkia*) up to 11.5% (*Hymenidium*). Note, that high level of intrageneric differences is often associated with polyphyly of the genus (6.8% in *Bunium*, 9.8% in

Trachydium). For example, in the phylogenetic tree representatives of the genus *Hymenidium* are placed in different clades (Fig. 4) located at considerable distances from one another. In case of monophyly of the genus, the level of differences is lower (5.3% in *Elaeosticta*, 3.0% in *Heracleum*, 2.8% in *Galagania*). However, when calculating the matrix of distances between sequences, homoplastic inversions were also taken into account, so the level of the differences may be even lower.

It was also revealed that lack of difference can be observed not only in sequences of different species of one genus (*Heracleum*, *Hyalolaena*, *Schrenkia*, *Bunium*, *Elaeosticta*, and *Galagania*), but also of sequences of species belonging to different genera and characterized by significant morphological differences (e.g. *Bunium afghanicum*, *Kosopoljanskia hebecarpa*, *Lipskya insignis*, and *Schtschurowskia meifolia*).

The revealed characteristics (high conservatism in closely related taxa, homoplastic inversions) cast doubt on the possibility of application of *psb*A-*trn*H for DNA barcoding of species in the family Umbelliferae. This once again emphasizes the fact that the laws of the evolution of the same region of the genome in different groups of plants may vary. Therefore, in the family Umbelliferae the *psb*A-*trn*H spacer can be of only limited use as a barcode.

Thus, on a large dataset of taxa of Umbelliferae characterized by various degrees of relationship, we were able to trace the changes occurring in the areas of the spacer under the influence of functional loading or without pronounced features. Still, the question remains whether the *trn*H gene has an independent promoter or that the gene is co-transcribed with *psb*A. The presence of large deletions in the spacer area adjacent to the *trn*H gene in the different groups of Umbelliferae supports this. Our data provide a good basis for further study of the functional characteristics of the *psb*A-*trn*H spacer in plants.

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APPENDIX

The GenBank accession numbers for sequences used in this work. The sequences obtained for the first time are marked with asterisks.

Tribe Pyramidoptereae (group 1). Group 1.1: group of species Bunium-I (*Bunium afghanicum* Beauverd EU445716, *B. angreni* Korovin EU445717, *B. badachschanicum* Kamelin EU445719, *B. capusii* (Franch.) Korovin EU445722, *B. caroides* (Boiss.) Hausskn. ex Bornm. EU445723, *B. chaerohylloides* (Regel & Schmalh.) Drude EU445724, *B. cylindricum* (Boiss. & Hohen. ex Boiss.) Drude EU445727, *B. fedtschenkoanum* Korovin ex Kamelin EU445729, *B. hissaricum* Korovin EU445732, *B. intermedium* Korovin EU445733, *B. kuhitangi* Nevski EU445735, *B. latilobum* Korovin EU445736, *B. longipes* Freyn EU445737, *B. persicum* (Boiss.) B. Fedtsch. EU445741, *B. salsum* Korovin EU445743, *B. seravschanicum* Korovin DQ457167, *B. setaceum* (Schrenk) H. Wolff DQ457168, *B. stewartianum* Nasir EU445734, *B. vaginatum* Korovin DQ457170, *B. wolffii* Kljuykov EU445746), *Elaeosticta alaica* (Lipsky) Kljuykov et al. HM474796, *E. allioides* (Regel & Schmalh.) Kljuykov et al. (1) HM474800, (2) HM474799, *E. buharica* (Korovin) Kljuykov et al. HM474801, *E. conica* Korovin HM474802, *E. ferganensis* (Lipsky) Kljuykov et al. (1) HM474805, (2) HM474806, (3) HM474807, *E. glaucescens* (DC.) Boiss. HM474808, *E. hirtula* (Regel & Schmalh.) Kljuykov et al. HM474810, *E. knorrigiana* (Korovin) Korovin EU445752, *E. korovinii* (Bobr. ex Korovin) Kljuykov et al. HM474813, *E. lutea* (M. Bieb. ex Hoffm.) Kljuykov et al. DQ457172, *E. meifolia* Fenzl HM474804, *E. nodosa* (Boiss.) Boiss. EU445753, *E. paniculata* (Korovin) Kljuykov & Pimenov HM474819, *E. platyphylla* (Korovin) Kljuykov et al. HM474820, *E. polycarpa* (Korovin) Kljuykov et al. HM474821, *E. ramosissima* Kljuykov HM474823, *E. samarkandica* (Korovin) Kljuykov et al. HM474824, *E. transcaspica* (Korovin) Kljuykov et al. HM474827, *E. transitoria* (Korovin) Kljuykov et al. HM474828, *E. tschimganica* (Korovin) Kljuykov et al. EU445754, *E. ugamica* (Korovin) Korovin HM474833, *E. vvedenskyi* (Kamelin) Kljuykov et al. HM474834, *Galagania ferganensis* (Korovin) M. Vassiljeva & Pimenov EU445756, *G. fragrantissima* Lipsky DQ457177, *G. gracilis* (Kamelin & Pimenov) Kamelin & Pimenov HM474835, *G. margiana* Pimenov & M. Vassiljeva HM474836, *G. neglecta* M. Vassiljeva & Pimenov HM474837, *G. platypoda* (Aitch. & Hemsl.) M. Vassiljeva & Pimenov HM474838, *G. tenuisecta* (Regel & Schmalh.) M. Vassiljeva & Pimenov DQ457176, *Hyalolaena bupleuroides* (Schrenk ex Fisch. & C. A. Mey.) Pimenov & Kljuykov EU445758, *H. depauperata* Korovin HM474840, *H. intermedia* Pimenov & Kljuykov HM474841, *H. jaxartica* Bunge EU445759, *H. lipskyi* (Korovin) Pimenov & Kljuykov HM474844, *H. transcaspica* (Korovin) Pimenov & Kljuykov HM474846, *H. trichophylla* (Schrenk) Pimenov & Kljuykov EU445760, *H. tschuiliensis* (Pavlov ex Korovin) Pimenov & Kljuykov EU445761, *H. viridiflora* Kljuykov HM474847, *Kosopoljanskia hebecarpa* Pimenov & Kamelin JF807602, *K. turkestanica* Korovin JF807603, *Lipskya insignis* (Lipsky) Nevski JF807604, *Mogoltavia sewerzowii* (Regel) Korovin DQ457178, *Oedibasis apiculata* (Kar. & Kir.) Koso-Pol. DQ457179, *Oe. platycarpa* (Lipsky) Koso-Pol. EU445763, *Oe. tamerlanii* (Lipsky) Korovin ex Nevski HM474850, *Pyramidoptera cabulica* Boiss. DQ457180, *Schrenkia congesta* Korovin JF807616, *Sch. fasciculata* Korovin JF807610, *Sch. golickeana* B. Fedtsch. JF807611, *Sch. involucrata* Regel & Schmalh. JF807612, *Sch. kultiassovii* Korovin JF807613, *Sch. papillaris* Regel & Schmalh. JF807614, *Sch. pungen* Regel & Schmalh. JF807617, *Sch. ugamica* Korovin JF807618, *Sch. vaginata* Fisch. & C. A. Mey. JF807619, *Schtschurowskia margaritae* Korovin JF807620, *Schtsch. meifolia* Regel & Schmalh. JF807621, *Schulzia albiflora* (Kar. & Kir.) Popov DQ457181, *Sch. crinita* (Pall.) Spreng. EU445770, *Sch. prostrata* Pimenov & Kljuykov HM474854, *Sclerotiaria pentaceros* (Korovin) Korovin JF807622. **Group 1.2:** group of species Bunium-II (*Bunium avromanum* (Boiss. & Hausskn. ex Boiss.) Drude74 EU445718, *B. balearicum* (Sennen) Mateo &S. Lypez Udias EU445720, *B. bulbocastanum* L. DQ457162, *B. bulbocastanum* L. var. *peucedanoides* (Desf.) J. M. Monts. EU445721, *B. cornigerum* (Boiss. & Hausskn. ex Boiss.) Drude EU445725, *B. corydalinum* DC. EU445726, *B. elegans* (Fenzl) Freyn DQ457163, *B. fallax* Freyn EU445728, *B. ferulaceum* Sm. EU445730, *B. fontanesii* (Pers.) Maire EU445731, *B. mauritanicum* Batt. EU445738, *B. microcarpum* (Boiss.) Freyn & Sint. ex Freyn DQ457164, *B. pachypodum* P. W. Ball EU445739, *B. paucifolium* DC. EU445740, *B. pinnatifolium* Kljuykov EU445742, *B. rectangulum* (Boiss. & Hausskn. ex Boiss.) H. Wolff DQ457166, *B. scabrellum* Korovin EU445744, *B. simplex* (K. Koch) Kljuykov DQ457169, *B. verruculosum* C. C. Townsend EU445745), *Postiella capillifolia* (Post ex Boiss.) Kljuykov EU445766, *Scaligeria napiformis* (Willd. ex Spreng.) Grande EU445768, *Tamamschjanella rhizomatica* (Hartvig) Pimenov & Kljuykov EU445774, *T. rubella* (E. Busch) Pimenov & Kljuykov EU445775.

Tribe Careae (group 2). Group 2.1: *Carum carvi* L. DQ457171, *C. caucasicum* Boiss. JF510478*, *C. meifolium* (M. Bieb.) Boiss. JF510480*, *Fuernrohria setifolia* K. Koch EU445755. **Group 2.2:** *Falcaria vulgaris* Bernh. DQ457174, *Olymposciadium caespitosum* Sibth. & Sm. JF807605. **Group 2.3:** *Aegopodium podagraria* L. EU445714, *A. alpestre* Ledeb. DQ457161.

Tribe Coriandreae (group 3): *Bifora testiculata* DC. JF807590, *B. radians* M. Bieb. JF807589, *Coriandrum sativum* L. JF807595.

Tribe Selineae (group 4): *Angelica apaensis* R. H. Shan & C. C. Yuan GU967807, *A. archangelica* L. EF590671, *A. dahurica* R. H. Shan & C. C. Yuan (1) EF590672, (2)

GQ435303, *A. decursiva* B. Fedtsch. GQ435319, *A. nitida* H. Wolff GU967809, *Peucedanum falcaria* Turcz. JF807607, *P. morisonii* Besser ex Schult. JF807608, *P. officinale* Besser JF807609, *Saposhnikovia divaricata* (Turcz.) Schischk. GQ435307, *Seseli montanum* L. JQ937303*.

Tribe Tordylieae (group 5). Group 5.1: *Heracleum anisactis* Boiss. & Hohen. ex Boiss. EF042113, *H. apiifolium* Boiss. DQ92728, *H. asperum* (Hoffm.) M. Bieb. DQ869376, *H. carpaticum* Porc. DQ927289, *H. cyclocarpum* K. Koch DQ996587, *H. dissectum* Ledeb. EF042117, *H. freynianum* Sommier & Levier DQ927291, *H. humile* Sm. DQ996575, *H. leskovii* Grossh. DQ869371, *H. ligusticifolium* M. Bieb. EF042115, *H. minimum* Lam. DQ927288, *H. pastinacifolium* K. Koch EF042116, *H. platytaenium* Boiss. DQ869373, *H. ponticum* (Lipsky) Schischk. ex Grossh. DQ869372, *H. rechingeri* Manden. DQ869370, *H. roseum* Steven DQ869375, *H. scabrum* Albov DQ869378, *H. sibiricum* L. DQ927290, *H. sosnowskyi* Manden. DQ869374, *H. trachyloma* Fisch. & C. A. Mey. DQ875445, *H. transcaucasicum* Manden. DQ869374, *Leiotulus porphyrodiscus* (Stapt & Wettst.) Pimenov & Ostroumova DQ927286, *L. secacul* (Mill.) Pimenov & Ostroumova DQ996578, *Mandenovia komarovii* (Manden.) Alava EF042110, *Pastinaca clausii* (Ledeb.) Calest. DQ869377, *Symphyoloma graveolens* C. A. Mey EF042112. **Group 5.2:** *Pastinacopsis glacialis* Golosk. JQ937310*, *Semenovia bucharica* (B. Fedtsch. ex Schischk.) Manden. JQ937314*, *S. dasycarpa* (Regel & Schmalh.) Korov. ex Pimenov & V. N. Tikhom. JQ937313*, *S. pimpinellioides* (Nevski) Manden. JQ937316*, *S. propinqua* (Aitch.) Manden. JQ937315*, *Tordyliopsis brunonis* DC. JQ937311*, *Zosima korovinii* Pimenov JQ937320*.

Tribe Scandiceae (group 6): *Anthriscus sylvestris* (L.) Hoffm. EU445715, *Caucalis platycarpos* L. JF807594, *Chaerophyllum aureum* L. EU445748, *Conopodium bourgaei* Coss. JQ937304, *C. majus* (Gouan) Loret. JQ937305, *Daucus aureus* Desf. EU531688, *D. carota* L. DQ898156, *D. setifolius* Kuntze EU531685, *Ferula communis* L. HE602477, *F. kingdon-wardii* H. Wolff JQ937319*, *F. kokanica* Regel & Schmalh. JQ937317*, *F. violaceae* Korovin JQ937318*, *Geocaryum bornmuelleri* (H. Wolff) Engstrand JQ937308*, *G. creticum* (Boiss. & Heldr.) Engstrand JQ937307*, *G. macrocarpum* (Boiss. & Spruner) Engstrand JQ937309*, *G. parnassicum* (Boiss. & Heldr.) Engstrand JQ937306*, *Myrrhis odorata* Scop. EU445762, *Osmorhiza longistylis* DC. DQ006137, *Scandix pecten-veneris* L. EU445769, *Thapsia platycarpa* Pomel HE659549, *Th. transtagana* Brot. HE659548, *Th. villosa* L. EU531683, *Torilis japonica* DC. FJ395453, *Turgenia latifolia* Hoffm. JF807624, *T. lisaeoides* C. C. Towns. JF807625.

Group of species Hymenidium-III (group 7): *Hymenidium chloroleucum* (Diels) Pimenov and Kljuykov FJ475143, *H. delavayi* (Franch.) Pimenov and Kljuykov FJ475146, *H. heterosciadium* (H. Wolff) Pimenov and Kljuykov FJ475149, *H. hookeri* (C. B. Clarke) Pimenov and Kljuykov FJ475150.

Clade Physospermopsis (group 8): group of species Hymenidium-II (*Hymenidium amabile* (Craib and W. W. Smith) Pimenov and Kljuykov FJ475137, *H. lhasanum* Pimenov and Kljuykov FJ475152, *H. nanum* (Rupr.) Pimenov and Kljuykov FJ475156, *H. virgatum* Pimenov and Kljuykov FJ475158), H*ymenolaena badachschanica* Pissjaukova FJ475161, *H. candollei* Wall. ex DC. FJ475162, *H. pimpinellifolia* Rupr. FJ475163, *Pterocyclus angelicoides* (DC.) Klotzsch FJ475173, *P. forrestii* (Diels) Pimenov and Kljuykov FJ483504, *Trachydium involucellatum* Shan & F. T. Pu FJ483509, *T. simplicifolium* W. W. Smith FJ475177, *T. souliei* H. Boissieu FJ475178, *T. variabile* H. Wolff HQ246206.

Tribe Pleurospermeae (group 9). Group 9.1: *Aulacospermum anomalum* (Ledeb.) Ledeb. FJ475132, *A. popovii* (Korovin) Kljuykov FJ475133, *A. simplex* Rupr. FJ475134, *A. stylosum* (C. B. Clarke) Rech. f. & Riedl FJ475135, group of species Hymenidium-I (*Hymenidium benthamii* (DC.) Pimenov and Kljuykov FJ475139, *H. davidii* (Franch.) Pimenov and Kljuykov FJ475144, *H. decurrens* (Franch.) Pimenov and Kljuykov FJ475145, *H. foetens* (Franch.) Pimenov and Kljuykov FJ475147, *H. hedinii* (Diels) Pimenov and Kljuykov FJ475148, *H. huzhihaoi* Pimenov and Kljuykov FJ475151, *H. lindleyanum* (Klotzsch) Pimenov and Kljuykov FJ475153, *H. linearilobum* (W. W. Smith) Pimenov and Kljuykov FJ475154, *H. stellatum* (D. Don) Pimenov and Kljuykov FJ475157, *H. wrightianum* (H. Boissieu) Pimenov and Kljuykov FJ475160, *H. wilsonii* (H. Boissieu) Pimenov and Kljuykov FJ475159), *Physospermopsis muktinathensis* Farille and S. B. Malla FJ475165, *Ph. nana* (Franch.) Pimenov and Kljuykov FJ475166, *Pleurospermum austriacum* (L.) Hoffm. FJ475168, *P. uralense* Hoffm. FJ475169, *Pseudotrachydium dichotomum* (Korovin) Pimenov and Kljuykov FJ475172, *P. kotschyi* (Boiss.) Pimenov and Kljuykov FJ475170, *P. vesiculoso-alatum* (Rech. f.) Pimenov and Kljuykov FJ475171, *Trachydium roylei* Lindl. FJ475176. **Group 9.2:** *Physospermum cornubiense* (L.) DC. EU445765, *Eleutherospermum cicutarium* Boiss. JQ937325*.

Tribe Bupleureae (group 10): *Bupleurum falcatum* L. HQ246208.

Tribe Heteromorpheae (group 11): *Anginon paniculatum* (Thunb.) B. L. Burtt JQ937324*, *Anginon swellendamense* (Eckl. & Zeyh.) B. L. Burtt JQ937323*, *Glia prolifera* (Burm. f.) B. L. Burtt HQ829358, *Heteromorpha arborescens* Cham. & Schltdl. HQ829359, *H. pubescens* Burtt Davy HQ829360.

Subfamily Saniculoideae (group 12): *Astrantia minor* L., *Eryngium alpinum* L. GQ385229, *E. campestre* L. HE602478, *E. giganteum* M. Bieb. FJ475136, *Sanicula europaea* L. FJ395459.

Subfamily Makinlayoideae (group 13): *Centella asiatica* (L.) Urb. (1) GQ435311, (2) GQ435309.

Family Araliaceae (group 14): *Hydrocotyle bonariensis* Lam. JQ937322*, *H. vulgaris* L. FM207082. **Group 15:** *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. GU054800, *Aralia elata* (Miq.) Seem. GU054875, *Hedera helix* L. AY163517, *H. nepalensis* K. Koch HM755916.