VERSITA

Biologia **65**/5: 805—812, 2010
Section Botany
DOI: 10.2478/s11756-010-0101-z

RFLP analysis of cpDNA in the genus *Hypericum*

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Abstract: The chloroplast DNA of 43 species including 16 sections from the genus *Hypericum* was studied by PCR-RFLP analysis. The PCR-amplified products of four cpDNA regions, *trn*C-*trn*D, *psb*C-*trn*S, *trn*L-*trn*F and *rbc*L were digested with four restriction endonucleases. A high level of interspecific variation was detected while intraspecific diversity was not observed. The resulting parsimony analysis indicated the monophyletic assemblage of the sections *Androsaemum*, *Olympia*, *Drosocarpium* and *Trigynobrathys*. Monophyly of *Hypericum* is weakly supported, but close relationships of *H. perforatum* and *H. maculatum* are indicated. The members of *Ascyreia* are weakly resolved, but clustering of *H. kouytchense* and *H. oblongifolium* is well supported, however, *H. reptans* is nested with *Olympia*. CpDNA profiles and the positions on the parsimony tree indicate that the chloroplast donor among the putative parents of the hybrid species *H.* ×*inodorum* is *H. androsaemum*.

Key words: *Hypericum*; cpDNA; PCR-RFLP; phylogeny

Introduction

Hypericum (Hypericaceae) is a large genus comprising about 450 species occurring in all temperate regions of the world. Most of these species can be easily recognized by their opposite simple leaves, often containing translucent and dark reddish or black glands, and by their 5-merous yellow flowers with numerous stamens assembled frequently in 3 or 5 bundles. In spite of these typical characters, wide-ranging interspecific variability (habitus, leaf size and shape, flower diameter and number, glands, venation) is found within the genus *Hypericum*. Robson (1977, 1981) reviewed and revised the previous classifications and suggested intrageneric subdivision into 30 main sections and 6 additional subsections based on extensive investigation, description and comparison of anatomical and morphological characters, ecology and distribution, cytological data and the results of phytochemical analyses (summarized in Robson 2003).

The economic importance of the genus *Hypericum* resides largely in a wide range of medicinal effects exhibited by *H. perforatum* L. such as anti-inflammatory, antiviral, antimicrobial, antifungal, cytotoxic and antidepressant activity. This important medicinal plant contains pharmacologically active compounds, such as naphthodianthrones, hypericin and pseudohypericin, phloroglucinols, hyperforin and adhyperforin, as well as characteristic xanthones, flavonoids, biflavonoids, tannins and phenolic acids (Barnes et al. 2001; Greeson et al. 2001). As important compounds occur in a few sections of the genus (Bruni & Sacchetti 2009), a considerable number of its species are likely to have useful pharmaceutical properties, which emphasizes the importance of this genus.

Despite the economical potential of the *Hypericum* species, there are only two references on the application of molecular markers in *Hypericum*: phylogenetic studies both employing nuclear rDNA ITS sequence data (Crockett et al. 2004; Park & Kim 2004). Preliminary PCR-RFLP analysis of cpDNA showed a considerable degree of interspecific polymorphism and confirmed the applicability of this method in phylogenetic investigations in the genus *Hypericum* (Hazler Pilepić 2002).

Apart from this, there are numerous publications on the application of molecular markers in *Hypericum.* Analysis of DNA variation in somaclones of *H. perforatum* was performed using restriction fragment length polymorphism (RFLP, Halušková & Čellárová 1997) and random amplified polymorphic DNA (RAPD, Halušková & Košuth 2003). Arnholdt-Schmitt (2000) investigated the mode of reproduction in *H. perforatum* using RAPD markers, Mayo & Langridge (2003) using multilocus genomic DNA RFLP and amplified fragment length polymorphism (AFLP). Intermicrosatellite (ISSR), RAPD or AFLP were applied to determine levels of genetic variation of natural *H. perforatum* populations (Barcaccia et al. 2006; Hazler Pilepić et al. 2008) or in comparison of natural *H. perforatum* populations with commercially cultivated ac-

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cessions (Percifield et al. 2007). Variable numbers of tandem repeats (VNTR) were used to distinguished apomictically and sexually derived plants in testing of hypericin content in *in vitro* regenerating plants and their seed progeny (Koperdakova et al. 2007). Nineteen variable microsatellite loci were developed for differentiation among individuals within and among populations of *H. cumulicola* (Edwards et al. 2007).

Here we present a molecular phylogenetic study of the genus *Hypericum* based on restriction fragment length polymorphism (RFLP) of four regions within the chloroplast genome of 43 species. The goals were to test (1) the taxonomic or phylogeographic implications of these cpDNA markers in the genus *Hypericum*, (2) if the morphology-based taxonomy is supported by cpDNA variation, and (3) the presumed maternal origin of two hybrid species (*H. perforatum* and *H.* ×*inodorum*).

Material and methods

Plant material and DNA extraction

278 accessions were included, involving 43 *Hypericum* species from 16 sections and *Triadenum walteri* (Gmelin) Gleason as an outgroup. Details about all plants studied are given in Table 1. The plants were cultivated in the Pharmaceutical Botanical Garden "Fran Kušan" of the Faculty of Pharmacy and Biochemistry, University of Zagreb. Voucher specimens are deposited in the Herbarium of the Department of Pharmaceutical Botany. Total cellular DNA was extracted from 100–140 g fresh or frozen leaves following the procedure of Doyle & Doyle (1990) modified as reported in Petit et al. (1993).

PCR-RFLP analysis

Total DNA was used as a template in PCR reactions with four universal chloroplast primer pairs (Hiratsuka et al. 1989; Taberlet et al. 1991; Demesure et al. 1995). The primer pairs used are listed in Table 2. Amplification reactions were performed in volumes of 25 μL containing 0.5 μM of each primer, 200 μM of each nucleotide, 2 mM $MgCl_2$ and 0.4 units of *Taq* DNA polymerase. Amplifications were performed as follows: first denaturation (4 min at 94 °C), 30 cycles of denaturation (45 s at 93 °C), annealing (45 s, T_{an} see Table 2), elongation (at 72 °C, T_{el} see Table 2) and final extension of 10 min at 72 °C. The amplified products were precipitated with ice-cold ethanol, washed with 70% ethanol and dissolved in water. The PCR products were verified by electrophoresis on 0.7% agarose gels containing ethidium bromide in Tris-acetate EDTA (TAE) buffer and detected under UV light. The size of the fragments was estimated by comparison with a molecular size standard (1 kb ladder, Gibco BRL).

The amplification products were digested with 2–4 four-base recognition restriction endonucleases (Table 2) for at least 4 hours at 37 °C or 65 °C (for *Taq*I). About 300 ng of the PCR products were digested in a 30 μL reaction mix according to the manufacturer's instructions (Invitrogen). The restriction fragments, along with a 100 bp ladder (Gene Ruler^{TM} 100bp ladder, Fermentas) as a size marker, were separated on 2% agarose gels in Tris-acetate EDTA (TAE) buffer and in 8% native acrylamide gels in Tris-borate EDTA (TBE) buffer, stained with ethidium bromide and photographed using an *ImageMaster* (VDS Pharmacia Tech) photodocumentation system. The identity of the restriction patterns with subtle differences in mobility were confirmed in duplicate experiments, running the samples of the respective taxa side by side in acrylamide gels, for longer periods of time. The interpretation of results is based on the restriction banding patterns observed without confirmation of band identity by Southern hybridization or sequencing. Considering the small taxonomic distance between the species, homology of all the comigrating bands was assumed. Both PCR amplification and restriction analysis of the PCR products were repeated at least twice for all samples.

Data analysis

Assuming that all studied cpDNA regions share a common evolutionary history, a data set matrix of restriction fragments was composed from ten electrophoretically well resolved PCR product/enzyme combinations listed in Table 3. Fragments were scored as binary characters (band presence/absence) for all corresponding band positions, across all samples and species. The data matrix is available from the corresponding author. Wagner parsimony analysis was performed on the obtained data matrix using the MIX program of the PHYLIP software package, version 3.6 (Felsenstein 2004), to construct the most parsimonious tree(s). The data were also bootstrapped to assess branch support, using SEQBOOT (1000 bootstrap replicates), followed by the MIX and CONSENSE programs of PHYLIP to obtain the consensus tree.

Results and discussion

PCR amplifications and restriction of PCR products

In the present study, three intergenic spacer regions: *trn*C-*trn*D (2600 bp), *psb*C-*trn*S (1600 bp), *trn*L-*trn*F (900 bp) and one gene region: *rbc*L (1300 bp) were analysed (a total length of approximately 6000 bp). No visually detectable variation was observed among the undigested PCR products following separation on agarose gel. We analysed 10 fragment/enzyme combinations and detected 110 different restriction patterns and 119 polymorphic fragments for the 43 *Hypericum* taxa studied (Table 3).

Figure 1 illustrates an example for the identity of *psb*C-*trn*S amplified products (a) and for the diversity of restriction patterns in the *psb*C-*trn*S/*Alu* I primer/restriction enzyme combination (b). The *trn*L-*trn*F fragment digested with *Alu* I showed the least polymorphism (4 fragments), the *trn*C-*trn*D fragment digested with *Taq* I exhibited the highest level of polymorphism (21 fragments).

Intraspecific polymorphism was neither detected in any of the *Hypericum* species nor in any of the cpDNA regions analysed. This result could be meaningful only for species *H. hirsutum*, *H. montanum and H. richeri* subsp. *grisebachii* since their numbers of individuals are relatively high and their provenance is from a few different locations (although all in Croatia). For other taxa, the unknown origin and the small number of individuals available to us did not permit rigorous analysis of intraspecific variation. We can, thus, not exclude some variation in some species, especially in those with

Table 1. *Hypericum* taxa collection information: taxon, number of sampled plants, geographic distribution, source of seeds, section [according to Robson (1981)].

Taxon	Number of samples	Geographic distribution	Source of seeds *	Section [Robson (1981)]
H. ascyron L.	5	Asia, N America	13	Roscyna (7)
H. androsaemum L.	7	Europe	9, 14	Androsaemum (5)
H. attenuatum Choisy	5	Asia	5	Hypericum (9)
H. balearicum L.	1	Balearic Islands	2	Psorophytum (2)
H. barbatum Jacq.	3	Balkan Peninsula	16	Drosocarpium (13)
H. bupleuroides Griseb.	5	NE Turkey	4	Bupleuroides (8)
H. calycinum L.	12	Bulgaria, Turkey	14	Ascyreia (3)
H. canariense L.	5	Canary Islands	6, 18	Webbia (21)
H. coris L.	1	Central Europe	17	Coridium (19)
H. delphicum Boiss & Heldr	5	Greece	2	Adenosepalum (27)
H. drummondii (Greve & Hook) Torrey & Gray	5	N America	19	Trigynobrathys (30)
H. dubium Leers	5	NW Europe	12	Hypericum (9)
H. foliosum Aiton	1	Azores	13	Androsaemum (5)
H. forrestii N. Robson	5	China, Burma	17	Ascyreia (3)
H. gentianoides (L.) Britton, Sterns & Pogg.	5	N America	19	Trigynobrathys (30)
H. hircinum L.	5	C & E Europe, Levant	17	Androsaemum (5)
H. hirsutum L.	16	Europe	13, 20	Taeniocarpium (18)
H. hookerianum Wight & Arnott	5	E Asia	7, 16	Ascyreia (3)
H. humifusum L.	5	W & C Europe	10	Oligostema (14)
H. hypericoides (L.) Crantz	5	America, Mexico	19	Myriandra (20)
H. × *inodorum* Miller	5	W Europe	1	Androsaemum (5)
H. kalmianum L.	5	N America	18	Myriandra (20)
H. kamtschaticum Ledeb.	5	Kamchatka	4	Hypericum (9)
H. kouytchense H.Lév.	5	China	17	Ascyreia (3)
H. maculatum Crantz	10	Europe, W Asia	11, 14	Hypericum (9)
H. montanum L.	14	Europe, W Asia (Krym)	20	Adenosepalum (27)
H. oblongifolium Choisy	5	India	10	Ascyreia (3)
H. olympicum L.	13	SE Balkans	9	Olympia (10)
H. orientale L.	5	NW Turkey, Caucasus	5, 18	Crossophyllum (16)
H. patulum Thunb. ex Murray	5	China, Taiwan, Japan	3	Ascyreia (3)
H. perforatum L.	15	Europe, Asia	14, 16, 20	Hypericum (9)
H. polyphyllum Boiss & Balansa	5	SW Asia	4	Olympia (10)
H. prolificum L.	3	NE America, Canada	3, 18	Myriandra (20)
H. pseudohenryi N. Robson	5	China	7	Ascyreia (3)
H. pulchrum L.	5	NW Europe	9	Taeniocarpium (18)
H. reptans Hook. F. & Thomson ex Dyer	5	China, Burma, India	8	Ascyreia (3)
H. reflexum L.	5	Canary Islands	2	Adenosepalum (27)
H. richeri Vill subsp. *grisebachii* (Boiss.) Nyman	15	C & S Europe	20	Drosocarpium (13)
H. stragulum Adams & Robson	5	NE America	19	Myriandra (20)
H. tetrapterum Fries.	17	Europe	9, 16	Hypericum (9)
H. triquetrifolium Turra	5	SE Mediterranean	16	Hypericum (9)
H. yezoense Maxim.	5	Asia	15	Hypericum (9)
H. wilsonii N. Robson	5	China	7	Ascyreia (3)
Outgroup *Triadenum walteri* (Gmelin) Gleason	5	America	19	

*1 – Botanischer Garten Heinrich-Heine-Universität, Düsseldorf, Germany; 2 – Botanischer Garten der Martin-Luther Universität, Halle, Germany; 3 – Royal Botanic Garden of Madrid, Spain; 4 – Botanical Garden of Šiauliai University, Šiauliai, Lithuania; 5 – Genebank, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany; 6 – Giadrini Botanici Hanbury, Genova, Italy; 7 – Hortus Botanicus Glasneviensis, Dublin, Ireland; 8 – Humboldt-Universität, Berlin, Germany; 9 – Jardin Botanique de la Ville et de l'Université, Cean, France; 10 – Jardin Botanique, Nantes, France; 11 – Kärntner Botanikzentrum, Klagenfurt, Austria; 12 – National Botanic Garden of Belgium, Meise, Belgium; 13 – Natural History Museum Botanical Garden, Oslo, Norway; 14 – Pharmaceutical Botanical Garden, Zagreb, Croatia; 15 – Research Center for Medicinal Plant Resources, Tsukuba, Japan; 16 – Royal Botanic Gardens, Kew, United Kingdom; 17 – Botanischer Garten, Ruhr-Universität Bochum, Bochum, Germany; 18 – Botanischer Garten,Universität Hohenheim, Stuttgart, Germany; 19 – Sparkleberry Springs, Georgia, USA, 20 – natural stands in Croatia.

wide geographical distribution and considerable morphological and ecological variability (*H. perforatum*, for example).

Cluster analysis of cpDNA data

Due to the complex digestion patterns, precise assignment of mutation types was not always possible. Thus, only the presence or absence of a restriction fragment was scored for all restriction patterns. Although restriction site analysis is often preferred in phylogenetic studies, comparative studies (Wang et al. 2000; van Droogenbroeck et al. 2004) show agreement with restriction fragment analysis. In fact, for example in *Vasconcellea*, fragment analysis showed even better resolution, likely due to the extra information derived from the length mutations (van Droogenbroeck et al. 2004). The length (insersion/deletion, or indel) mutations, which, in a number of published studies (Amane et al. 1999; Mohanty et al. 2001; Dane et al. 2004), accounted for most of the observed cpDNA variation, are

Table 2. CpDNA primer pairs (1 – Demesure *et al.* 1995, 2 – Taberlet *et al.* 1991, 3 – Hiratsuka *et al.* 1989) and the respective annealing temperatures (T_{an}) and elongation times (T_{el}) used during the PCR reactions. Size of the products and restriction endonucleases used for digestion are shown.

Primer pair/reference	T_{an}/°C	T_{el}/min	Product size (bp)	Restriction enzyme
*trn*C-*trn*D/1	58.0	4	2300	*Hinf* I, *Taq* I
*psb*C-*trn*S/1	57.0	2	1600	*Alu* I, *Hae* III, *Hinf* I, *Taq* I
*trn*L-*trn*F/2	50.0	1	900	*Alu* I, *Taq* I
*rbc*L/3	50.0	1	1300	*Alu* I, *Hae* III

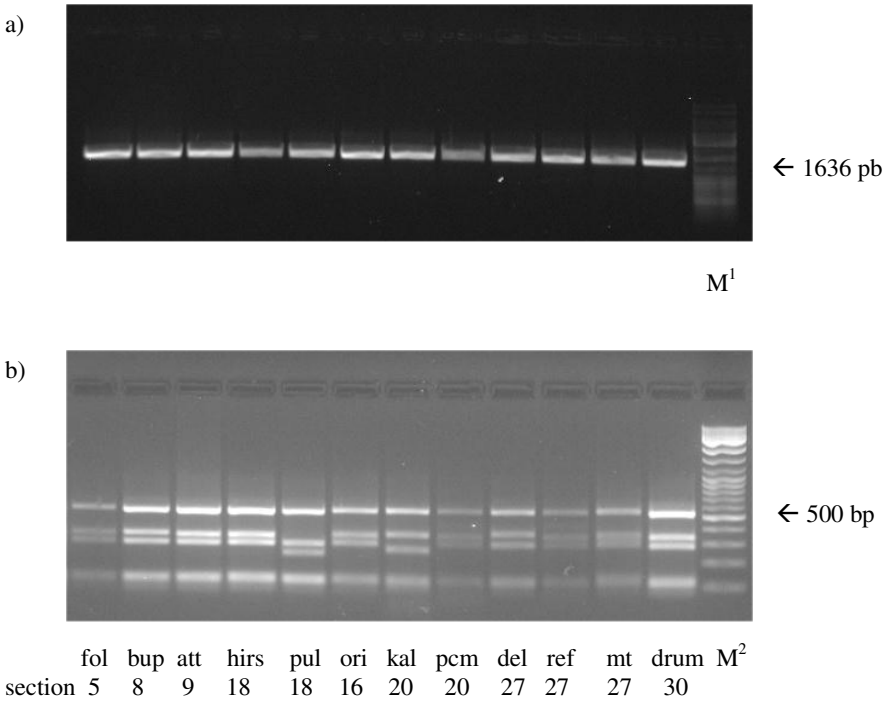

Fig. 1. a – *psb*C-*trn*S PCR products, and b – restriction fragments after digestion with *Alu* I for 12 *Hypericum* species (fol – *H. foliosum*, bup – *H. bupleuroides*, att – *H. attenuatum*, hirs – *H. hirsutum*, pul – *H. pulchrum*, ori – *H. orientale*, kal – *H. kalmanium*, pcm – *H. prolificum*, del -*H. delphicum*, ref – *H. reflexum*, mt – *H. montanum*, drum – *H. drummondii*, M^1 – 1 kb ladder, Gibco BRL marker, M^2 – Gene Ruler^{TM} 100 bp DNA Ladder marker). Sections: 5 – *Androsaemum*, 8 – *Bupleuroides*, 9 – *Hypericum*, 18 – *Taeniocarpium*, 16 – *Crossophyllum*, 20 – *Myriandra*, 27 – *Adenosepalum*, 30 – *Trigynobrathys.*

often omitted from the majority of data analyses, thus discarding possibly useful information (Bremer 1991). In our results, the slight differences in fragment size of the bands with similar mobility, which were detected in all restriction profiles of the studied *Hypericum* species, could be explained by indel mutations. These bands were used as informative characters in our data analysis.

Parsimony analysis, based on Wagner's method, resolved 56 equally most parsimonious trees with a length of 395 steps. The 50% majority rule consensus tree is shown in Fig. 2. The tree shows eight clades. The first clade (I, bts = 97%) comprises the species *Hypericum hypericoides* and *H. stragulum* from section *Myriandra*. *H. hypericoides* is widely distributed and a very variable species. *H. stragulum* Adams & Robson (syn. *H. hypericoides* subsp. *multicaule* (Michx. ex Willd.) N Robson) is considered as one of the three subspecies of *H. hypericoides.* From the large section *Myriandra* we investigated another two taxa:*H. kalmianum* and *H. prolificum.* They fell into another clade with high bootstrap support (VII, bts = 100%) confirming the close relationship of these two species, which are distinguished only in some morphological details. According to Robson's genealogy of section *Myriandra* (1996), the very variable *H. prolificum* is considered the pivotal species, while *H. kalmianum* has derived characters. The segregation of *Myriandra* in our tree is in accordance with subdivision into five subsections defined on morphological characters such as number of petals, sepals and styles, type of placentation and habit (Robson, 1996). According to this subdivision, *H. kalmianum* and *H. prolificum* belong to subsect. *Centrosperma* while *H. hypericoides* and *H. stragulum* belong to subsect. *Ascyrum.* On the contrary, ITS sequence data proved the monophyly of *Myriandra* (Crockett et al. 2004). The inconsistencies between gene trees based on nuclear and plastid markers are not uncommon and could be attributed to lineage sorting, hybridization / reticulation, nonhomologous sampling of duplicated gene, or chloroplast capture (Hendy & Penny 1989; Doyle 1997; Maddison 1997; Sang & Zhong 2000; Tsitrone et al. 2003;

Table 3. Summary of the fragments detected by PCR-RFLP. The numbers given for each fragment/enzyme combination indicate the number of polymorphic restriction fragments and the number of patterns.

Cp DNA region	Restriction enzyme			
	Alu I	*Hae* III	*Hinf* I	*Taq* I
*trn*C-*trn*D	–	–	13, 21	21, 21
*psb*C-*trn*S	11, 10	19, 14	11, 10	10, 7
*trn*L-*trn*F	4, 3	–	–	7, 4
*rbc*L	12, 11	11, 9	–	–
Total = 119, 110	27, 24	30, 23	24, 31	38,32

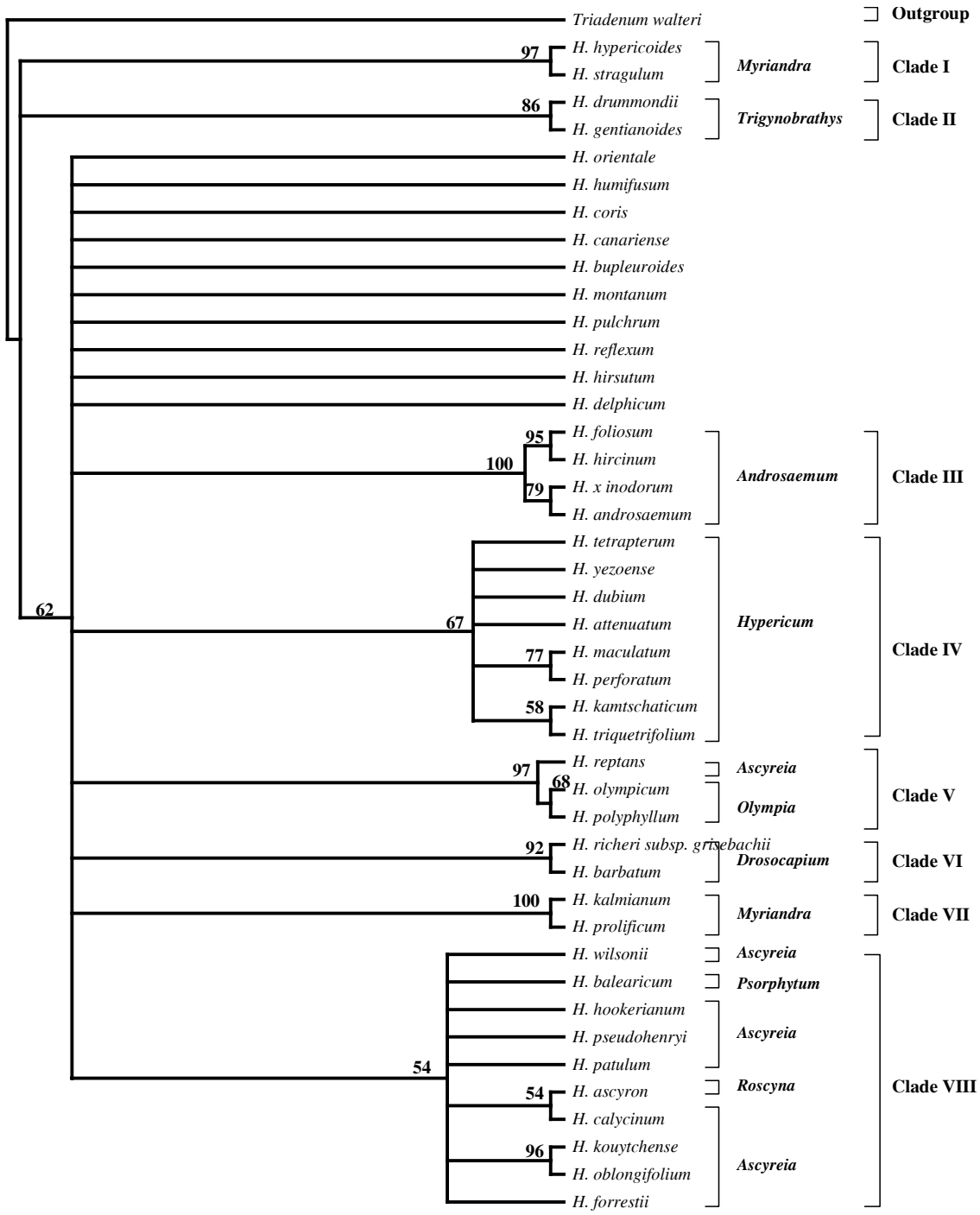

Fig. 2. The 50% majority rule consensus tree based on 119 polymorphic restriction fragments of four cpDNA regions for 43 *Hypericum* species. The bootstrapping percentages (support higher than 50% is shown) from 1000 replicates are given alongside the branches.

Okuyama et al. 2005). For this reason Okuyama et al. (2005) suggested using of multiple molecular tools that have the potential to reveal detailed organismal evolution processes.

ITS data (Park & Kim 2004) suggested a monophyletic origin of section *Trigynobrathys* (section 30), in agreement with Robson (1990) who proposed the African *H. revolutum* subsp. *keniense* as the progenitor. The two representatives of this section in our data set, *H. drummondii* and *H. gentianoides*, form a well supported clade in the cpDNA tree (II, bts = 86%) supporting their distinct position in relation to the other species in the sample and the monophyletic origin of this section.

Four taxa in our study belong to *Androsaemum* (section 5): *H. foliosum*, *H. androsaemum*, *H. hircinum*, and the spontaneous hybrid of the latter, *H.* ×*inodorum*. These four species share several morphological properties and the same geographic distribution. Our cpDNA tree confirms the monophyletic origin of section 5 with high support (clade III, bts = 100%), and *H. androsaemum* as a chloroplast donor in the origin of *H.* ×*inodorum* (bts = 79%).

The large section *Hypericum* (section 9) comprises 48 species and has 8 representatives in our work which are clustered together, although with low support (clade IV, bts = 67). The taxa investigated belong to the series *Hypericum* of sect. *Hypericum* which is related to *H. ascyron* subsp. a*scyron* (Robson 2002), a concept not confirmed by our results. *H. perforatum* and *H. maculatum* are clustered together (bts = 77%) indicating their close relationship which was proved recently by cytogenetic analysis of *H. perforatum*, *H. maculatum* and *H. attenuatum* (Brutovská et al. 2000). The results of this study indicate a difference of *H. attenuatum* in one gene locus in comparison with the other two species which argue against a theory about a hybrid origin of *H. perforatum* from *H. attenuatum* (Robson 1981; Campbell & Delfosse 1984) and suggest that this species could have evolved from *H. maculatum* directly or from their common ancestor. *H. attenuatum*, the most primitive Asian species, is the basic species of the *H. yezoense* group (Robson 2002). Kitamura & Murati (1962) place these two species in the same group due to their morphological similarities, but on the cpDNA tree, *H. attenuatum* and *H. yezoense* are unresolved. *H. kamtschaticum* is also closely related to *H. attenuatum* (Park 1974; Ohwi 1984) but, on our cpDNA tree, *H. kamtschaticum* forms a low supported branch (bts = 58%) with the Eastern Mediterranean species *H. triquetrifolium*. An ITS sequence phylogeny including 20 mostly Asian species clearly showed section 9 to be a polyphyletic assemblage (Park & Kim 2004). The authors suggested two evolutionary lineages of the Asian species from the section *Hypericum* which might be confirmed by plastid DNA analyses applied on the Asian set of taxa.

Within the weakly supported clade VIII (bts = 54%), the majority of taxa from section *Ascyreia* (section 3) are nested together with representatives of sections *Roscyna* (*H. ascyron,* section 7) and *Psorophytum* (*H. balearicum*, section 2) indicated some relation of these three sections. Only the mutual relations of the *H. kouytchense* and *H. oblongifolium* are highly supported (bts = 96). *H. kouytchense* seems to be one of the most primitive species in *Ascyreia* (long styles and stamens, acute leaves and sepals, large stellate flowers and conical ovary and capsule, Robson 1985). For both *H. kouytchense* and *H. oblongifolium*, variation in chromosome number from diploid to tetraploid has been recorded, and both are facultative apomicts as is the case for all species from *Ascyreia* in our sample except for *H. reptans*, which is a sexual species (Matzk et al. 2003). Interestingly, in our cpDNA tree, *H. reptans* has an appearance quite different from that of the other species in the section *Ascyreia*. Separation of this obligate sexual diploid gives some evidence that chloroplast markers may provide important insights into hybridization and polyploidy, two common evolutionary processes in *Hypericum*. *H. reptans* is a morphologically well-characterized taxon with some peculiarities in comparison with others in our sample such as a particular habitus (prostrate shrublet) and very small leaves and flowers. It is joined with species from sections *Olympia* (section 10) in clade V (bts = 97%), *H. olympicum* and *H. polyphyllum.* They are related species from the SE Balkan Peninsula and Turkey, and are the only members of *Olympia*. The position of these species on the cpDNA tree corroborates the current phylogenetic position and suggests some relationship with *H. reptans.*

Two Balkan species, *H. richeri* Vill subsp. *grisebachii* (syn. *H. alpigenum* Kit.) and *H. barbatum*, belong to *Drosocarpium*, section 13. Our cpDNA analysis confirms their relationship (clade VI, bts = 92%).

Ten species (Fig. 2) form a multifurcation along with clades III-VIII probably caused by insufficient accumulation of informative variation confirming lower cpDNA sequence variability. On the other hand, the investigated regions have been used extensively for plant molecular phylogenetic analyses at species level and above. Additional cpDNA regions should be tested for better resolution of all species.

We believe that the grouping seen in our analysis may reflect some geographic pattern. Since *Hypericum*, *Myriandra and Trigynobrathys* are the very large and widely distributed sections, biogeographic structuring of cpDNA haplotypes might be present. Similar cpDNA RFLP profiles of species sharing a common geographic range (*H. perforatum* and *H. maculatum, H. hypericoides* and *H. stragulum*, *H. kalmianum* and *H. prolificum*, *H. drummondii* and *H. gentianoides*, members of*Androsaemum* or *Drosocarpium*) may have biogeographical impact, but for final conclusions, a comparison with other members of these sections as well as with other *Hypericum* species from these geographic ranges is necessary.

Hypericum is one of about hundred large plant genera which are notoriously difficult for taxonomic study due to their size alone. Beside, *Hypericum* has

very ancient members as well as species that are regarded as evolutionarily young, which often are products of interspecific hybridization. Many of them reproduce apomictically now and have different ploidy levels. In addition, the very wide geographic distribution of the entire genus and some of its members may stimulate the formation of different cpDNA haplotypes. All of these aspects influence the choice of markers. Chloroplast markers for lower-level systematic studies are especially needed, because data from the usually maternally inherited chloroplast genome and limited recombination of cpDNA can provide important insights into hybridization and polyploidy, two common evolutionary processes in plants, as well as into current biogeographical structuring and therefore historical patterns of species evolution (Chester et al. 2007). The present study employed four polymorphic chloroplast DNA regions (*trn*C-*trn*D, *psb*C-*trn*S, *trn*L-*trn*F and *rbc*L). Although the resolution and sampling in this current study is in some cases not sufficient to permit major conclusions of phylogeny and biogeography in *Hypericum*, the results partially corroborate previous classifications of *Hypericum* species, suggest some of novel relations and provide some clues for further studies in this genus. As pointed out by Wolfe & Liston (1998) and Wang et al. (2000), PCR-RFLPs can be used as a selection for informative regions to be subjected to detailed sequence analysis. Comparisons between phylogenies inferred from both nuclear and chloroplast genomes would provide a better basis for assessing species relationships in *Hypericum*.

Acknowledgements

The authors would like to thank N. Ljubešić and H. Fulgosi from the Laboratory of Electron Microscopy, Division of Molecular Biology at the Ruđer Bošković Institute for kindly providing laboratory facilities. We are also grateful to all institutions which helped us with seeds from their collections. We also thank V. Magnus for helpful suggestions and critically reading the manuscript and reviewers for their constructive comments and suggestions to improve the manuscript. This research was supported by The Croatian Ministry of Science, Education and Sports, Projects no. 006-222 and 006-0061117-1239.

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Received June 18, 2009
Accepted December 9, 2009