



# Low rate of point mutation in the mtDNA noncoding fragment of *Hypericum* taxa

KROATA HAZLER PILEPIĆ<sup>1</sup>  
IVA BOŽIĆ<sup>1</sup>  
ANTONELA LEKIĆ<sup>1</sup>  
ZLATKO LIBER<sup>2</sup>  
ŽELJAN MALEŠ<sup>1</sup>  
IVANA MARKIĆ<sup>1</sup>  
MARIJA TABAK<sup>1</sup>  
VANJA ŽUVELA<sup>1</sup>

<sup>1</sup>Department of Pharmaceutical Botany  
Faculty of Pharmacy and Biochemistry  
University of Zagreb  
Schrott-ova 39, 10 000 Zagreb, Croatia

<sup>2</sup>Botanical Department and Botanical  
Garden, Division of Biology  
Faculty of Science, University of Zagreb  
Marulićev trg 9a, 10000 Zagreb, Croatia

## Correspondence:

Kroata Hazler Pilepić  
Department of Pharmaceutical Botany  
Faculty of Pharmacy and Biochemistry  
University of Zagreb  
Schrott-ova 39, 10 000 Zagreb, Croatia  
E-mail: tina@pharma.hr

**Key words:** *Hypericum*, mtDNA RFLP,  
phylogeny

## Abstract

**Background and Purpose:** Contrary to a major impact of phylogenetic studies on humans and animals, application of the mitochondrial DNA (mtDNA) analysis in plant phylogeny research is rather sporadic due to its high rate of rearrangements and low rate of point mutations. Nevertheless, mtDNA analysis gave interesting results in some taxa. With the aim to test variability and applicability of the mtDNA in the phylogenetic investigation of genus *Hypericum*, four noncoding regions of mtDNA were compared using the mtDNA RFLP on thirty-six *Hypericum* taxa.

**Materials and Methods:** Total cellular DNA was extracted from fresh or frozen leaves of thirty-six *Hypericum* taxa native in different part of the world. The PCR-amplified products of four noncoding mtDNA regions (*ccb*, *cox3*, *nad1*, *nad4*) were digested with six restriction endonucleases.

**Results and Conclusions:** Out of thirty-six *Hypericum* taxa belonging to fifteen sections and 14 restriction profiles, differences were observed in only two species profile. One deletion was found in the *nad1/Hinf I* restriction profile of *H. pseudohenryi*, while gain of restriction site was detected in the *nad1/RsaI* restriction profile of *H. hirsutum*. Obtained results confirmed slow sequence evolution of the *Hypericum* mtDNA, for plant mtDNA in general.

## INTRODUCTION

The detection of appropriate molecular marker is of great importance for phylogeny and population studies. Contrary to cpDNA genetics which has been extensively used for plant molecular phylogenetic analysis, the plant mitochondrial DNA fragments have only rarely been used as a source of phylogenetic markers because of their presumed slow rate of nucleotide substitution (12, 13) and very high degree of variability in size and structure (20). Nevertheless, mtDNA analysis gave interesting results in some plants genera (7, 11, 19).

*Hypericum* (*Hypericaceae*) is a large genus comprising about 450 species occurring in all temperate regions of the world. Robson reviewed and revised the previous *Hypericum* intrageneric classifications (summarized in 18). Recently, cpDNA RFLP analysis (8, 9) and ITS sequence data (2, 10, 15) have been employed for molecular phylogenetic studies in *Hypericum*. Although the resolution and sampling in these studies are not sufficient to permit major conclusions on phylogeny and biogeography in *Hypericum*, the results partially corroborate actual classifications on *Hypericum* species. Still, the interest in popu-

TABLE 1

Hypericum taxa collection information: taxon, geographic distribution, source of seeds, section (17).

Taxon	Geographic distribution	Source of seeds *	Section
<i>H. ascyron</i> L.	Asia, N America	12	Roscyna (7)
<i>H. androsaemum</i> L.	Europe	9	Androsaemum (5)
<i>H. attenuatum</i> Choisy	Asia	5	Hypericum (9)
<i>H. barbatum</i> Jacq.	Balkan Peninsula	14	Drosocarpium (13)
<i>H. bupleuroides</i> Griseb.	NE Turkey	4	Bupleuroides (8)
<i>H. calycinum</i> L.	Bulgaria, Turkey	13	Ascyreia (3)
<i>H. canariense</i> L.	Canary Islands	6	Webbia (21)
<i>H. coris</i> L.	Central Europe	15	Coridium (19)
<i>H. delphicum</i> Boiss & Heldr	Greece	2	Adenosepalum (27)
<i>H. foliosum</i> Aiton	Azores	12	Androsaemum (5)
<i>H. forrestii</i> N. Robson	China, Burma	15	Ascyreia (3)
<i>H. hircinum</i> L.	C & E Europe, Levant	15	Androsaemum (5)
<i>H. hirsutum</i> L.	Europe	12	Taeniocarpium (18)
<i>H. hookerianum</i> Wight & Arnott	E Asia	7	Ascyreia (3)
<i>H. humifusum</i> L.	W & C Europe	10	Oligostema (14)
<i>H. × inodorum</i> Miller	W Europe	1	Androsaemum (5)
<i>H. japonicum</i> Thunb. ex Murray (syn <i>H. chinense</i> )	China, Korea, Japan	6	Trigynobrathys (30)
<i>H. kalmianum</i> L.	N America	16	Myriandra (20)
<i>H. kamtschaticum</i> Ledeb.	Kamchatka	4	Hypericum (9)
<i>H. kouytchense</i> H.Lév.	China	15	Ascyreia (3)
<i>H. linarifolium</i>	W Europe	15	Oligostema (14)
<i>H. maculatum</i> Crantz	Europe, W Asia	11	Hypericum (9)
<i>H. montanum</i> L.	Europe, W Asia (Krym)	17	Adenosepalum (27)
<i>H. oblongifolium</i> Choisy	India	10	Ascyreia (3)
<i>H. olympicum</i> L.	SE Balkans	9	Olympia (10)
<i>H. orientale</i> L.	NW Turkey, Caucasus	5	Crossophyllum (16)
<i>H. patulum</i> Thunb. ex Murray	China, Taiwan, Japan	3	Ascyreia (3)
<i>H. perforatum</i> L.	Europe, Asia	17	Hypericum (9)
<i>H. polyphyllum</i> Boiss & Balansa	SW Asia	4	Olympia (10)
<i>H. prolificum</i> L.	NE America, Canada	3	Myriandra (20)
<i>H. pseudohenryi</i> N. Robson	China	7	Ascyreia (3)
<i>H. pulchrum</i> L.	NW Europe	9	Taeniocarpium (18)
<i>H. reptans</i> Hook. F. & Thomson ex Dyer	China, Burma, India	8	Ascyreia (3)
<i>H. reflexum</i> L.	Canary Islands	2	Adenosepalum (27)
<i>H. richeri</i> Vill subsp. <i>grisebachii</i> (Boiss.) Nyman	C & S Europe	17	Drosocarpium (13)
<i>H. tetrapterum</i> Fries.	Europe	9	Hypericum (9)

\*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, Caen, France; 10 – Jardin Botanique, Nantes, France; 11 – Kärntner Botanikzentrum, Klagenfurt, Austria; 12 – Natural History Museum Botanical Garden, Oslo, Norway; 13 – Pharmaceutical Botanical Garden, Zagreb, Croatia; 14 – Royal Botanic Gardens, Kew, United Kingdom; 15 – Botanischer Garten, Ruhr-Universität Bochum, Bochum, Germany; 16 – Botanischer Garten, Universität Hohenheim, Stuttgart, Germany; 17 – natural stands in Croatia.

lation genetics and molecular phylogeny is growing for using and combining several different molecular markers, with the aim to obtain more information and produce better conclusions. The availability of a great number of universal primers capable of amplifying specific regions of mitochondrial genomes (3, 5, 6) by using the polymerase chain reaction (PCR) has made it possible to explore the diversity of the organelle DNA for taxonomic and phylogenetic purposes. Restriction fragment length polymorphism (RFLP) is a useful screening method for variability of target fragments, which often precedes sequencing. To our knowledge, there are no published reports on the mtDNA fragment analysis in *Hypericum* species.

The objectives of this study were: (1) to test whether the published universal primers of mtDNA could amplify respective non-coding regions in *Hypericum* spp., (2) to detect a nucleotide substitution in a large number of *Hypericum* spp. from distant regions of the world, (3) to test the applicability of the mtDNA regions in *Hypericum* phylogenetic reconstruction.

## MATERIALS AND METHODS

### Plant material

Thirty-six *Hypericum* species from fifteen sections were included in the study. 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 (4) modified as reported in Petit *et al.* (16).

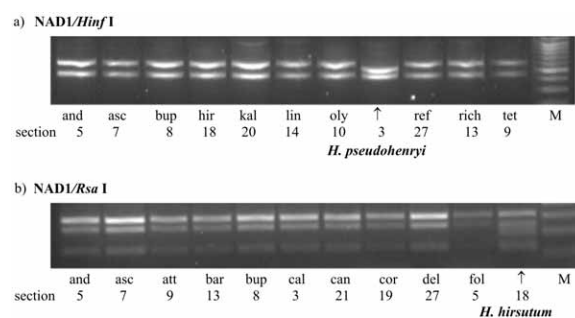
### PCR-RFLP analysis

Total DNA was used as a template in PCR reactions with four universal mitochondrial primer pairs (TABLE 2). Amplification reactions were performed in volumes of 100 µl containing 0.5 µM of each primer, 200 µM of each nucleotide, 2 mM MgCl<sub>2</sub> and 0.4 units of *Taq* DNA polymerase. Amplifications were performed using the following conditions: first denaturation (4 min at 94°C), 30 cycles of denaturation (45 s at 92°C), annealing (45 s, T<sub>an</sub> see Table 2), elongation (at 72°C, T<sub>el</sub> see Table 2) and

final extension for 10 min at 72°C. 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 (Gene Ruler™ 100bp ladder, Fermentas). The amplification products were digested with 6 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 manufacturer's instructions (Invitrogen). The restriction fragments, along with a 100 bp ladder (Gene Ruler™ 100bp ladder, Fermentas) as a molecular size marker, were separated on 2% agarose gels in Tris-acetate EDTA (TAE) buffer, stained with ethidium bromide and photographed using an *ImageMaster* (VDS Pharmacia Tech) photodocumentation system.

## RESULTS AND DISCUSSION

In the present study, four noncoding regions: *ccb* (500 bp), *cox3* (700 bp), *nad1* (1200 bp) and *nad4* (2000 bp) were analyzed (a total length of approximately 4400 bp).



**Figure 1.** Detected polymorphism in restriction fragments of *nad1* introne after digestion with *Hinf* I (a) and *Rsa* I (b) for some *Hypericum* species (and – *H. androsaemum*, asc – *H. ascyron*, att – *H. attenuatum*, bar – *H. barbatum*, bup – *H. bupleuroides*, cal – *H. calycinum*, can – *H. canariense*, cor – *H. coris*, del – *H. delphicum*, fol – *H. foliosum*, hir – *H. hirsutum*, kal – *H. kalmanium*, lin – *H. linarifolium*, oly – *H. olympicum*, ref – *H. reflexum*, rich – *H. richeri*, tet – *H. tetrapterum*, M – Gene Ruler™ 100bp ladder, Fermentas). Sections: 3 – *Ascyreia*, 5 – *Androsaemum*, 7 – *Roscyra*, 8 – *Bupleuroides*, 9 – *Hypericum*, 10 – *Olympia*, 13 – *Drosocarpium*, 14 – *Oligostema*, 18 – *Taeniocarpium*, 19 – *Coridium*, 20 – *Myriandra*, 21 – *Webbia*, 27 – *Adenosepalum*.

**TABLE 2**

The respective annealing temperatures (T<sub>an</sub>) of the mtDNA fragments and their elongation times (T<sub>el</sub>) used in the PCR reactions, size of the products and respective restriction endonucleases used for digestion are shown below.

Fragment / reference	T <sub>an</sub> /°C	T <sub>el</sub> /min	Product size (bp)	Restriction enzyme
<i>ccb</i> / 5	57.0	1	500	<i>Alu</i> I, <i>Hinf</i> I, <i>Taq</i> I
<i>cox3</i> / 5	57.0	1	700	<i>Alu</i> I, <i>Hae</i> III, <i>Hinf</i> I, <i>Taq</i> I
<i>nad1</i> / 3	57.5	2	1200	<i>Alu</i> I, <i>Hae</i> III, <i>Hinf</i> I, <i>Hpa</i> I, <i>Rsa</i> I
<i>nad4</i> / 3	57.5	2	2000	<i>Alu</i> I, <i>Hinf</i> I

TABLE 3

Summary of the polymorphism detected after restriction of mtDNA fragments: + detected polymorphism, 0 nondetected polymorphism, – nonrestricted or unspecific digestion.

mtDNA fragment	Polymorphism / number of digestion products					
	<i>Alu I</i>	<i>Hae III</i>	<i>Hinf I</i>	<i>Hpa I</i>	<i>Rsa I</i>	<i>Taq I</i>
ccb	0 / 2	–	0 / 2	–	–	0 / 2
cox3	0 / 2	0 / 2	0 / 2	–	–	0 / 2
nad1	0 / 5	0 / 5	+ / 2	0 / 2	+ / 3,4	–
nad4	0 / 5	–	0 / 2	–	–	–

No visually detectable variation was observed among the undigested PCR products following separation on agarose gel. We analyzed 14 fragment/enzyme combinations (Table 3). Out of the thirty-six *Hypericum* taxa, belonging to fifteen sections, a difference in profiles of only two species was observed in the nad1 intron. One deletion was found in the nad1 fragment of *H. pseudohenryi* after digestion with *Hinf I* (Figure 1a). The gain of a restriction site was detected in the nad1 fragment of *H. hirsutum*, after digestion with *Rsa I* (Figure 1b). The observed insufficient accumulation of informative variation confirms lower mtDNA sequence variability. The sequence evolution appears to be quite slow in the plant mtDNAs in general, with exception of the *Pelargonium* and *Plantago* species where sequence evolution seems to be strikingly accelerated (1, 14). The studied mtDNA regions are not suitable to be used as molecular markers for *Hypericum* phylogenetic analyses. Moreover, we concluded that geographic isolation by distance does not show any notable influence on the mtDNA structure in *Hypericum*. However, additional mtDNA regions should be tested.

**Acknowledgements:** The authors would like to thank Prof Dr. sc. 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 provided us with seeds from their collections. This research was supported by the Croatian Ministry of Science, Education and Sports Project No. 006-006117-1239.

## REFERENCES

- BAKKER F T, CULHAM A, PANKHURST C E, GIBBY M 2000 Mitochondrial and chloroplast DNA-based phylogeny of *Pelargonium* (Geraniaceae). *Am Jour Bot* 87: 727–734
- CROCKETT S L, DOUGLAS A W, SCHEFFLER B E, KHAN I A 2004 Genetic profiling of *Hypericum* (St. John's Wort) species by nuclear ribosomal ITS sequence analysis. *Planta Med* 70: 929–935
- DEMASURE B, SODZI N, PETIT R J 1995 A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol Ecol* 4: 129–131
- DOYLE J J, DOYLE J L 1990 Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15
- DUMINIL J, PEMONGE M H, PETIT R J 2002 A set of 35 consensus primer pairs amplifying genes and introns of plant mitochondrial DNA. *Mol Ecol Notes* 2: 428–430
- DUMOLIN-LAPEGUE S, PEMONGE M-H, PETIT R J 1997 An enlarged set of consensus primers for the study of organelle DNA in plants. *Mol Ecol* 6: 393–397
- EDWARDS E J, NYFFELER R, DONOGHUE M J 2005 Basal cactus phylogeny: implications of *Pereskia* (Cactaceae) paraphyly for the transition to the cactus life form. *Am Jour Bot* 92: 1177–1188
- HAZLER PILEPIĆ K 2002. Analysis of chloroplast DNA variability among some *Hypericum* species in Croatia. *Period biol* 104: 457–462
- HAZLER PILEPIĆ K, MOROVIĆ M, ORAL F, ŠANTOR M, VEJNOVIĆ V 2010 RFLP analysis of cpDNA in the genus *Hypericum*. *Biologia (Bratislava)* 65: 805–812
- HAZLER PILEPIĆ K, BALIĆ M, BLAŽINA N 2011 Estimation of phylogenetic relationships among some *Hypericum* (Hypericaceae) species using ITS sequences. *Plant Biosys* 145: (article in press)
- HU D, LUO Z 2006 Polymorphisms of amplified mitochondrial DNA non-coding regions in *Diospyros* spp. *Sci Hort* 109: 275–281
- KNOOP V 2004 The mitochondrial DNA of land plants: peculiarities in phylogenetic perspective. *Curr Genet* 46: 123–139
- PALMER J D, SOLTIS D, SOLTIS P 1992 Large size and complex structure of mitochondrial DNA in two nonflowering land plants. *Curr Genet* 21: 125–129
- PALMER J D, ADAMS K L, CHO Y, PARKINSON C L, QIU Y L, SONG K 2000 Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutations rates. *Proc Natl Acad Sci USA* 97: 6960–6966
- PARK S J, KIM K J 2004 Molecular phylogeny of the genus *Hypericum* (Hypericaceae) from Korea and Japan: evidence from nuclear rDNA ITS sequence data. *J Plant Biol* 47: 366–374
- PETIT R J, KREMER A, WAGNER D B 1993 Geographic structure of chloroplast DNA polymorphisms in European oaks. *Theor Appl Genet* 87: 122–128
- ROBSON N K B 1981 Studies in the genus *Hypericum* L. (Guttiferae). 2. Characters of the genus. *Bull Br Mus Nat Hist (Bot)* 8: 55–226
- ROBSON N K B 2003 *Hypericum* botany. In: Edzard E (ed) *Hypericum*. Taylor & Francis, London, New York, p 1–23
- WEI W, YOU LIANG Z, LI C, YUMING W, ZEHONG Y, RUIWU Y 2005 PCR-RFLP analysis of cpDNA and mtDNA in the genus *Houttuynia* in some areas of China. *Hereditas* 142: 24–32
- WOLFE K H, LI W H, SHARP P M 1987 Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proc Natl Acad Sci USA* 88: 9054–9058