

Taxonomic individuality of *Leonurus cardiaca* and *Leonurus quinquelobatus* in view of morphological and molecular studies

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Abstract The main goal of this study was to determine the number and taxonomic rank of taxa belonging to the complex *Leonurus cardiaca* agg. in Poland. Based on statistical analysis of selected features, two morphological forms of this plant were distinguished. In order to determine their genetic polymorphism and the relationships between them, the nuclear, mitochondrial and chloroplast genomes were analysed with the use of RAPD and PCR–RFLP markers. 39 RAPD primers produced a total of 234 nuclear DNA fragments, of which 128 were polymorphic and distributed almost equally between two forms. It was found that 87 % of the compared pairs of RAPD profiles differ from each other. Five chloroplast and two mitochondrial primer pairs were used to amplify non-coding regions of organelle genomes. Restriction analysis revealed uniformity of mtDNA and occurrence of two cpDNA haplotypes, corresponding to naked and hairy forms of *L. cardiaca* agg. The obtained results justifies the recognition of these forms as separate species *L. cardiaca* s. s. L. and *L. quinquelobatus* Gilib., respectively. The distribution of both species in Poland is given in the paper.

Keywords Lamiaceae · *Leonurus* · Taxonomy · Differentiation · RAPD · RLFP · cpDNA · mtDNA

Introduction

Leonurus cardiaca L. agg. is recognised as one of the most valuable melliferous and herbal plants (Koltowski 2006; Popescu et al. 2009). Two or three morphological forms, whose taxonomic rank is not established yet, can be distinguished within the complex. They are considered separate species, subspecies or varieties of *L. cardiaca* (Holub 1993; Kretovskaja 1989, 1990; Govaerts et al. 2011, Mirek et al. 2002; Tomšowic 2000). It is not known whether particular forms have similar apiarian value and the content of biologically active substances.

The strongly haired form was first distinguished from broadly understood *L. cardiaca* and described by Gilibert (1793) as a separate species *L. quinquelobatus* Gilib. The name agrees with ICBN and as a priority should be valid. Hence, the later name *L. villosus* Desf. ex d’Urv. and based on it *L. cardiaca* subsp. *villosus* (Desf. ex d’Urv.) Hyl. and *L. cardiaca* var. *villosus* (Desf. ex d’Urv.) Nyman should be dealt with as synonyms. Now, the name *L. quinquelobatus* is accepted in the World Checklist (Govaerts et al. 2011) and by Russian authors (Kretovskaja 1989, 1990; Troeva et al. 2010).

Holub (1993) described the species named *L. intermedius* Holub, a transitory form between *L. cardiaca* s. str. and *L. quinquelobatus* (= *L. villosus*). The taxon, however, is not accepted in the rank of species now, even by Czech authors (Tomšowic 2000) and is treated as a synonym of *L. cardiaca* s. str. (Govaerts et al. 2011). This arises some doubts since the diagnostic features of *L. intermedius* indicated by Holub (1993) fall rather within the range of

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variability of *L. quinquelobatus* and so *L. intermedius* should be treated as a synonym of species described by Gilibert. In general, identification of different forms within this group, based on morphology or chemical features is difficult and controversial; this may result from the susceptibility of these traits to environmental influences. In such a case, studies on the nuclear and/or organellar DNA level may give better results.

There are a number of strategies available for detecting nuclear DNA variation in plants, of which randomly amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990) has proved particularly useful because of their simplicity and cost-effectiveness. RAPD markers are employed widely in population studies, cultivar identification and in analysis of hybrid individuals (Stiles et al. 1993; Huang et al. 2000; Lind-Halldèn et al. 2002; Abraham et al. 2010). Despite the slower rate of sequence evolution in organelles, intraspecific variation has been reported for cpDNA in populations of trees such as *Fagus sylvatica* L. (Demesure et al. 1996), *Quercus robur* L. (Dumolin-Lapègue et al. 1997, 1998), *Prunus avium* L. (Mohanty et al. 2001), *Prunus spinosa* L. (Mohanty et al. 2003), herbs (Gielly and Taberlet 1994; Gielly et al. 1996; Stewart et al. 2011) or crop plants (Chen et al. 1993). Focusing on non-coding regions usually extend the utility of cpDNA at lower taxonomic levels (Gielly and Taberlet 1994). Such regions seem to be particularly useful for studying phylogenetic relationships between closely related species, and for genetic population studies at both intra- and inter-specific level (Demesure et al. 1996; Dumolin-Lapègue and Petit 1997). In some studies, mitochondrial DNA (mtDNA) variations are similarly informative (Van Droogenbroeck et al. 2006). In most angiosperms, chloroplast and mitochondrial genomes are inherited maternally (Reboud and Zeyl 1994; Dumolin-Lapègue et al. 1995) and, therefore, are often investigated simultaneously.

The analyses of nuclear DNA (ITS) and chloroplast DNA (matK) in Chinese representatives of *Leonurus* indicated that molecular studies may be helpful in solving taxonomic problems in this genus (Zhi-Ye et al. 2011). In view of the still existing disagreement as to the rank of *Leonurus* taxa described above, we decided to compare them on molecular level using nuclear (RAPD) and organelle (PCR-RFLP) DNA markers. To our knowledge, these studies are the first approach to solve taxonomic ambiguities within the *L. cardiaca* agg. complex at molecular level.

We attempted in our study to answer the following questions: (1) how species of the complex *L. cardiaca* agg. are distributed in Poland, (2) is there a genetic reason for the individuality of *L. cardiaca* s. str. and *L. quinquelobatus* at the species level, and (3) is it possible to distinguish an intermediate taxon between them (respective to *L. intermedius*) based on analysis of morphological features.

Materials and methods

Plant material deposited in Polish herbaria (acronyms of herbaria acc. to Mirek et al. 1997) and own materials were used in the first part of the study (analyses of morphological features). Maps of plant distribution in the ATPOL system of squares of a side of 10 km (Zajac and Zajac 2001) were based exclusively on the own materials and verified herbarium specimens.

Morphometric studies

The following parameters were determined in 248 measured herbarium specimens:

1. density and distribution of stem hairs—hairs adpressed, spread, hairs dense, sparse, stem wholly haired, only on edges,
2. length of stem hairs in mm,
3. hairs on the lower side of leaves—sparse, dense; length of hairs in mm,
4. size and shape of lower leaves—maximum length and maximum width (cm), the number of lobes, the depth of leaf blade indentations,
5. calyx morphology—hairs (naked, scarcely, densely haired), length of hairs (in mm), length of the calyx (in mm), length of the calyx teeth (in mm).

100 specimens were selected for statistical analyses: 44 specimens of *L. cardiaca* s. str., 35 of *L. quinquelobatus* and 21 specimens of intermediate features corresponding to the description of *L. intermedius* (Holub 1993). Original data of a mixed character i.e. measurements, binary data (0/1) and rank order (missing, rarely, densely) were standardised with the PragmaTax (Moraczewski 2009) programme to the range [0, 1]. Based on so prepared data, Manhattan distance was calculated with the PragmaTax software and then a dendrogram was constructed with the UPGMA method.

RAPD analysis

Total genomic DNA was extracted from seedlings of *L. cardiaca* and *L. quinquelobatus*, obtained from seeds collected in sites in Nowogród and Korczew, using a cetyl trimethyl ammonium bromide (CTAB) method (Williams et al. 1990). Analyses were carried out through RAPD markers using bulk samples of genomic DNA (BSA) as a template. PCR amplification was performed in volume of 16 µl containing 8 µl a ready-to-use PCR Master Mix (2×) (Fermentas), 20 pM 10-mer primer and about 50 ng of template DNA. Forty 10-nucleotide primers from commercially available kits A and H (Operon Technologies Inc., USA) were tested in the study. DNA amplification

Table 1 Pairs of primers used to amplify *cpDNA* regions in *L. cardiaca* and *L. quinquelobatus*

Primer 1	Primer 2	Abbreviations	References
<i>trnC</i> [tRNA–Cys (GCA)]	<i>trnD</i> [tRNA–Asp (GUC)]	CD	Demesure et al. (1995)
<i>trnK</i> [tRNA–Lys (UUU)exon 1]	<i>trnK</i> [tRNA–Lys (UUU)exon 2]	K1K2	Demesure et al. (1995)
<i>trnS</i> [tRNA–Ser (GGA)]	<i>trnT</i> [tRNA–Thr (UGU)]	ST	Demesure et al. (1995)
<i>trnV</i> [tRNA–Val (UAC)3'exon]	<i>rbcL</i> [RuBisCO large subunit]	VL	Dumolin–Lapègue et al. (1997)
<i>trnF</i> [tRNA–Phe (GAA)]	<i>trnL</i> [(UAA) 5'exon]	FL	Taberlet et al. (1991)

reactions were performed in a thermocycler (M.J. Research, Inc.) programmed for 40 cycles divided into two stages differing in terms of annealing temperature. After initial denaturation at 95 °C for 300 s, each cycle was composed of denaturation step at 92 °C for 90 s, annealing step for 90 s (35 °C for first 20 cycles and 38 °C for the next 20 cycles) and extension step at 72 °C for 120 s. The reaction was completed by final synthesis at 72 °C for 300 s and storage at 4 °C until turned off.

The amplified products were separated by electrophoresis in 1.5 % agarose gels containing ethidium bromide, in 1× TBE buffer, in the presence of size markers and photographed under UV light.

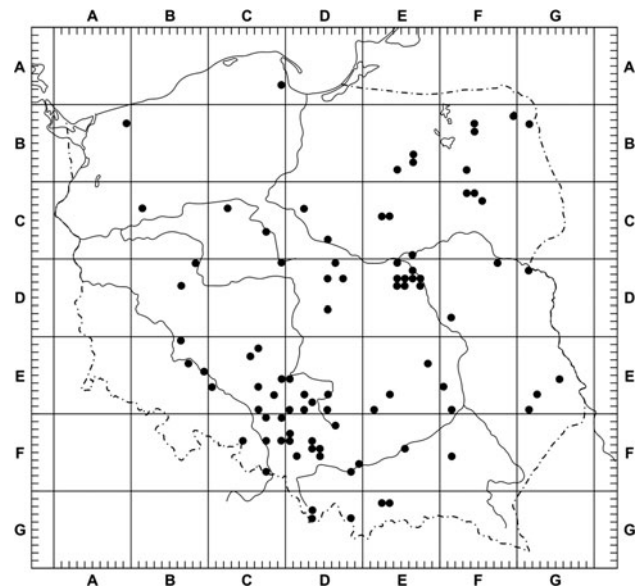
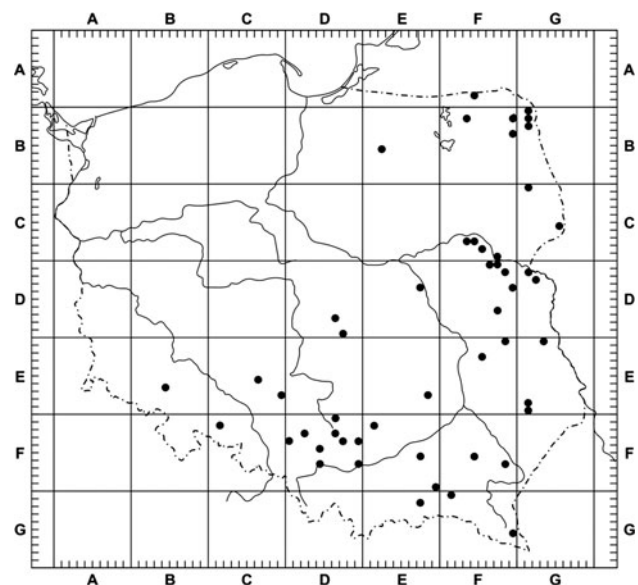
PCR–RFLP analysis

Five chloroplast and two mitochondrial primer pairs, previously tested for different plant species and described by Demesure et al. (1995), Dumolin–Lapègue and Petit (1997) and Taberlet et al. (1991), were used to amplify non-coding regions (Table 1). All reactions were performed in 25 µl containing 10× PCR buffer and 1.5 units of Tag polymerase (Sigma), 7.5 nM dNTP, 10 pmol each primer and 50 ng total DNA. After initial denaturation at 95 °C for 5 min, PCR was performed for 30 cycles, each consisting of 93 °C, 30 s, 45 s at annealing temperature (57.5 °C for the regions *trnV–rbcL* and *trnC–trnD* or at 53 °C for *trnF–trnL*, *trnS–trnT*, *trnK1–trnK2*, *orf25* and *cox1*) and synthesis at 72 °C (2 min for *cpDNA*, 1 min for *mtDNA*).

Table 2 *cpDNA* regions and restriction enzymes used for hydrolysis of PCR amplified *cpDNA* fragments

The amplified regions of <i>cpDNA</i>	The applied restriction enzymes
<i>trnC–trnD</i>	<i>HinfI</i> , <i>HindIII</i> , <i>DraI</i> , <i>MboI</i> , <i>AluI</i>
<i>trnS–trnT</i>	<i>HinfI</i> , <i>AluI</i> , <i>Sau3A</i> , <i>DraI</i>
<i>trnK–trnK</i>	<i>HinfI</i> , <i>HindIII</i> , <i>MvaI</i> , <i>PstI</i> , <i>MspI</i> , <i>SmaI</i> , <i>MboI</i>
<i>trnF–trnL</i>	<i>HinfI</i> , <i>AluI</i> , <i>Sau3A</i> , <i>MboI</i> , <i>PstI</i> , <i>DraI</i> , <i>SmaI</i> , <i>TagI</i>
<i>trnV–rbcL</i>	<i>MvaI</i> , <i>MboI</i> , <i>MspI</i>

Enzymes which gave polymorphic pattern are underlined

**Fig. 1** Distribution of *Leonurus cardiaca* s.str. in Poland, verified by the authors**Fig. 2** Distribution of *Leonurus quinquelobatus* in Poland, verified by the authors

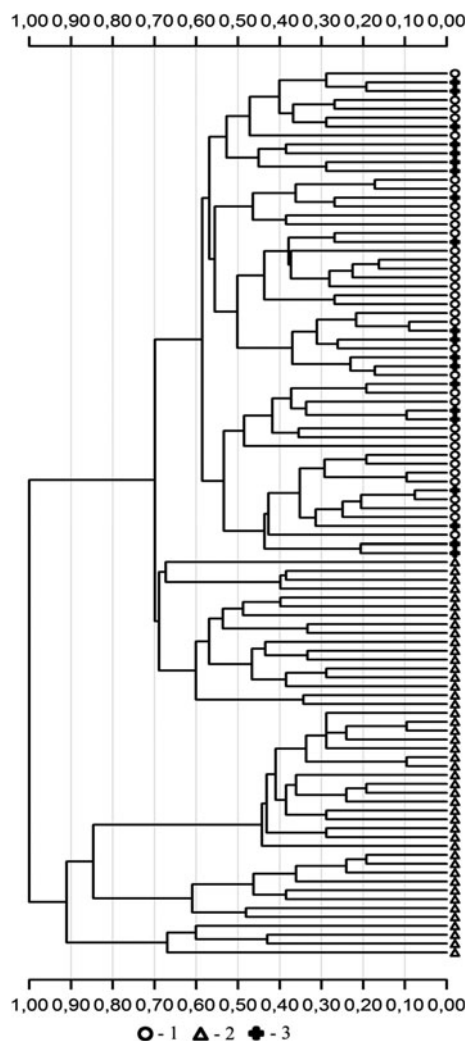
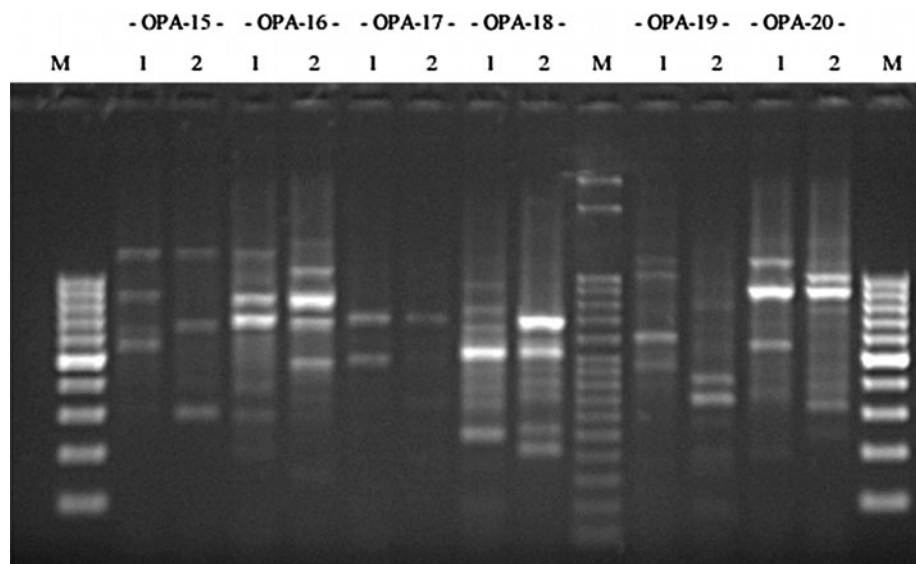


Fig. 3 UPGMA dendrogram of morphological differentiation of *Leonurus cardiaca* s. l., 1 *Leonurus quinquelobatus*, 2 *L. cardiaca* s. str., 3 *L. intermedius*

Fig. 4 RAPD profiles of *Leonurus cardiaca* (1) and *L. quinquelobatus* (2) using primers indicated above each pair plants analysed. M molecular markers 100 and 50 bp



Final extension was at 72 °C for 10 min. For restriction digestion, 6–10 µl of PCR products were used. Restriction endonucleases used in this study are listed in Table 2. Restriction fragments were separated in 8 % polyacrylamide gel, stained in the ethidium bromide solution and photographed under UV light.

Results

Maps of the distribution of *L. cardiaca* s. str. and *L. quinquelobatus* in Poland are shown on Figs. 1, 2. The *L. cardiaca* complex occurs probably in the whole Poland's area being rare in the east and particularly in the north-east of the country. *Leonurus quinquelobatus* is the species of eastern range. In Poland it is common in the east, to the west it is being replaced with *L. cardiaca* s. str., and most probably occurs in this part of country only rarely.

Comparative analysis of morphological features of *L. cardiaca* s. str., *L. quinquelobatus* and hypothetical intermediate form (*L. intermedius* Holub) revealed the existence of only two clearly different groups (Fig. 3). One of them included exclusively those individuals of *L. cardiaca* s. str., which are characterised by naked stem or the stem covered by short adpressed hairs only on edges, and by the calyx naked or covered with a few short hairs. The second group consisted of individuals belonging to the typical *L. quinquelobatus* (whole plants densely and long haired), intermediate forms (stems, calyxes and leaves more or less covered by hairs of a length intermediate between those of *L. cardiaca* and *L. quinquelobatus*), and a small well-distinguished subgroup composed of individuals of *L. cardiaca* with typically haired stem and densely and long haired calyx.

A total of 40 decamer oligonucleotide primers were used to investigate molecular differences between *L. cardiaca* s. str. and *L. quinquelobatus* on the nuclear DNA level. Besides one (OPH-10), they produced distinct banding patterns, composed of clear and readable bands. A total of 234 products were generated, of which 128 were polymorphic and distributed almost equally between two forms (62 and 66 polymorphic products in *L. cardiaca* s. str. and *L. quinquelobatus*, respectively). Analysis of fingerprints obtained for particular primers (Fig. 4) indicate distinct differences between two analysed taxa; 87 % of the compared pairs of DNA profiles differ from each other.

In most cases, the universal primers for cpDNA (FL, CD, VL, ST and K1K2) and mtDNA regions (*orf25* and *coxI*) produced fragments similar to those described by Demesure et al. (1995), Dumolin-Lapègue and Petit (1997) and Taberlet et al. (1991). With K1K2 primers two additional products were obtained, and with primers for *coxI* the produced mtDNA fragment was much larger than expected. The lengths of obtained amplification products did not differ between analysed plants.

No differences between the studied taxa were found after hydrolysis of mitochondrial DNA fragments by enzymes *AluI*, *HindIII*, *TagI* and *Sau3A*. Similar restriction fragments were also obtained for the *trnV-rbcL* region of cpDNA. In other PCR products, restriction analysis revealed a length polymorphism, most in *trnF-trnL* cpDNA (Fig. 5). There were also digestion products

Table 3 Variants of polymorphic fragments (in bp) obtained with combination of primer and different enzymes in *Leonurus*

Polymorphic fragments	<i>L. cardiaca</i>	<i>L. quinquelobatus</i>
FL- <i>HinfI</i>	0	155
FL- <i>MboI</i>	642	618
FL- <i>MboI</i>	769	748
FL- <i>MboI</i>	0	450
FL- <i>DraI</i>	719	667
FL- <i>DraI</i>	0	597
FL- <i>Sau3A</i>	656	628
FL- <i>Sau3A</i>	0	450
CD- <i>MboI</i>	727	770
ST- <i>HinfI</i>	471	492
K1K2- <i>MspI</i>	362	406
K1K2- <i>MspI</i>	424	485
K1K2- <i>MvaI</i>	215	0
K1K2- <i>HindIII</i>	238	0
K1K2- <i>HinfI</i>	252	0
K1K2- <i>PstI</i>	239	0

present only in the profiles of *L. quinquelobatus* and absent from *L. cardiaca*, or vice versa (e.g. *trnK1-trnK2*) (Table 3). The presence of polymorphic fragments the majority of analysed cpDNA regions indicate the existence of clearly different haplotypes in analysed forms.

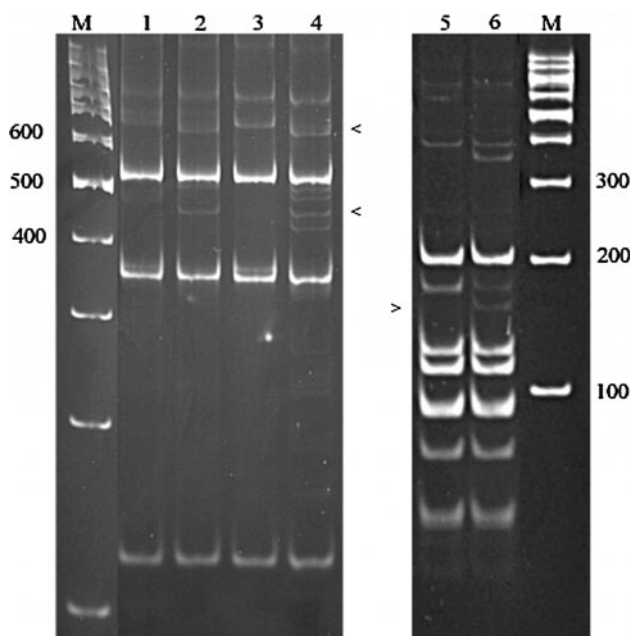


Fig. 5 Restriction patterns of primer-enzyme combinations FL-*Sau3A* (1–2), FL-*MboI* (3–4) and FL-*HinfI* (5–6) resolved in 8 % polyacrylamide gel stained with ethidium bromide. Polymorphic sites are indicated by arrows. *M* molecular size marker 100 bp ladder, 1,3,5—*L. cardiaca*; 2,4,6—*L. quinquelobatus*

Discussion

Results of our morphological analyses showed the existence of significant differences between typical forms of *L. cardiaca* s. str. and typical forms of *L. quinquelobatus*, which argues for recognising them as different species. A similar approach was used by Holub (1993), Kretovskaja (1989, 1990) and Govaerts et al. (2011). The typical form of *L. cardiaca* is characterised by very scarce, adpressed hair cover of the stem (hairs present only on margins), leaves and calyx, hairs are shorter than 0.5 mm and the plants are seemingly naked.

Typical *L. quinquelobatus* is characterised by dense spreading hairs which are longer than 1 mm. Morphological individuality may, however, raise some doubts due to the existence of intermediate forms. Although the dendrogram (Fig. 3) shows the discontinuity that divides the studied individuals into two groups, it does not, however, form a precise dividing line between *L. cardiaca* and *L. quinquelobatus*. The problem is in the subgroup of 17 specimens characterised by haired stem (hairs adpressed and present only on margins) and densely haired calyx, provisionally determined as *L. cardiaca*. Their morphological features correspond to *L. cardiaca* var. *hirtella*

described by Holub (1993). Considering that the basic feature discriminating *L. cardiaca* from *L. quinquelobatus* is the type of hair cover on the stem, one should identify the individuals of the stem haired only on margins and of haired calyxes with *L. cardiaca* despite the fact that they refer to *L. quinquelobatus*. On the other hand, intermediate forms corresponding to *L. intermedius* (Holub 1993) fall entirely within the range of variability of *L. quinquelobatus* and do not form a well-distinguished group. Hence, it appears that *L. intermedius* is a synonym of *L. quinquelobatus* and not of *L. cardiaca* as it was proposed by other authors (Govaerts et al. 2011). To resolve controversies around proper classification of two basic morphological forms of *L. cardiaca* agg., analyses at molecular level were performed.

The reliability of molecular studies is higher when supported by the use of various markers and different methods of analysis. Studies on phylogenetic relationships between cultivated and wild species of rice with the RAPD, RFLP and SSLP markers carried out by Bautista et al. (2001) or between yams and soybean species with the use of the RFLP, RAPD, AFLP and SSR markers (Powell et al. 1996; Mignouna et al. 2003) may serve as examples. In this study, parallel to the PCR–RAPD analysis of total DNA, PCR–RFLP of organelle genomes was used as a second method. Fingerprint analysis of nuclear DNA showed a high level of polymorphism between *L. cardiaca* s. s. and *L. quinquelobatus* and justifies their individuality determined on the basis of morphology. A similar conclusion can be drawn from the cpDNA analysis. Primers designed by Demesure et al. (1996) and Dumolin-Lapègue et al. (1997) successfully amplified five cpDNA regions, producing DNA fragments of the expected length in all analysed *Leonurus* plants. The polymorphisms detected after digestion of four of these fragments (*trnC–trnD*, *trnS–trnT*, *trnF–trnL* and *trnK1–trnK2*) clearly indicated the existence of different haplotypes in *L. cardiaca* s. s. and *L. quinquelobatus*. On the other hand, no differences were detected with respect to the length of DNA fragments obtained after amplification and hydrolysis of their mtDNA.

In summary, the morphological differences combined with DNA diversification clearly suggest the distinctiveness of two basal morphological forms of *L. cardiaca* agg. and justify the recognition of these forms as separate species, *L. cardiaca* s. s. L. and *L. quinquelobatus* Gilib. The forms of intermediate morphology will need, however, additional studies which will be possible after collecting more material.

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