DE GRUYTER OPEN DNA Barcodes 2015; Volume 3: 17–26

Rapid Communication **Open Access**

M. Combik*, Z. Mirek

Estimating the effectiveness of species identification by sequencing of two chloroplast DNA loci (matK and rbcL) in selected groups of Polish flora

Abstract: This paper focuses on assessing the effectiveness of species identification using barcoding standard loci (matK and rbcL) for a selected group of Polish flora. 55 samples of 17 species from six taxonomic groups representing various parts of the system, including Monocots and Dicots as well as Pteridophytes, were selected. PCR amplification success was 100% (of all samples) for the rbcL primer and 64% for matK primers giving in sum 87 fragments for sequencing. The aligned sequences show that in the *Vaccinium, Dryopters* and *Symphytum* species groups barcoding is very useful and makes it possible to identify most species in those groups. In the *Symphytum* genus only the rbcL locus shows differences between the species. In the case of *Dryopteris* due to problems with matK amplification only the rbcL locus could be analyzed, but it is highly useful in this group. Out of 17 species selected for this study, barcoding makes it possible to identify 11 of them, i.e. 64% of all. In the *Anthoxanthum* group, sequences of plastid loci show intraspecific variability which can be used to make phylogeographical analysis but could complicate species identification.

Keywords: barcoding effectiveness, polish flora, Monocots, Dicots, Pteridophytes, direct PCR

Doi: 10.1515/dna-2015-0003
received October 7, 2014 accepted February 3, 2015

***Corresponding author: M. Combik:** Władysław Szafer Institute of Botany, Polish Academy of Sciences, Kraków,
E-mail: albero@kki.pl,m.combik@botany.pl
Z. Mirek: Władysław Szafer Institute of Botany, Polish Academy of Sciences, Kraków

© 2015 M. Combik, Z. Mirek licensee De Gruyter Open.
This work is licensed under the Creative Commons Attribution-NonCommercial-NoDerivs 3.0 License.

1 Introduction

DNA barcoding is broadly used within the world of animals where DNA sequence of the Cox 1 gene is successfully used.

Unfortunately, in the case of plants we know that information from a single locus does not allow for the identification of plant species around the world. Despite these difficulties, numerous attempts to establish barcoding standards in plants were made and finally in 2009 the Consortium for the Barcode of Life Plant Working Group (CBOL) evaluated seven chloroplast genomic regions across the plant kingdom and proposed a combination of matK and rbcL as plant barcodes [1,2] It was considered that the sequences obtained from the matK and rbcL loci are the most universal and can be used as a standard.

Nucleotide sequences retrieved from these standard loci are compared with sequences from a previously created reference database which contains sequences obtained from herbarium voucher specimens.

It is clear that the wider application of barcoding requires a reference database containing sequence information about local flora.

The Władysław Szafer Institute of Botany is part of four Polish institutions which have been designated to develop DNA barcoding in Poland.

This paper is an attempt to evaluate if the loci considered as standard in DNA plants barcoding are useful for identifying species in selected groups of systematic Polish flora.

2 Material and Methods

Samples of 17 species from six taxonomic groups representing various parts of the system, including both

Monocots and Dicots as well as Pteridophytes, were selected as a good representation of vascular plant diversity. In *Vaccinium* genus four species were selected

Unauthenticated
Download Date | 10/21/17 1:28 AM

what is 4 of 4 native (polish) species and two samples from each species were tested. In *Dryopteris* genus four samples for *expansa* and *dilatata*, two for *carthusiana* and *filix-mas* and three for *borerrei* species were taken. In this genus 5 of 8 native species were selected. For the other genera each species were represented by 4 samples what it gives altogether 55 samples.

In *Anthoxanthum* and *Rumex* genus 2 of 3 native species were evaluated. In case of *Empetrum* 2 of 2 native species were selected. Lowest number of native species were tested in *Symphytum* genus were 2 of 4 species were chosen (Table 1). They enable testing the usability of the selected loci. The selected species include both very well defined and easily distinguishable species and so called microspecies which are difficult to distinguish on the basis of their morphological features.

The samples were dried and stored in silica gel immediately after being collected as fresh green tissue. To simplify analysis procedure and minimize costs, the direct PCR method was used to obtain the template for DNA sequencing directly from the dried sample. Direct PCR was made using Phire Plant Direct PCR Kit (Thermo Scientific) in accordance with manufacturer's recommendations. Small fragments of dried samples (0.5 sq mm) were suspended into 20ul of a "dilution buffer" by mixing thoroughly. This solution was used as the DNA template in direct PCR. The primers used in PCR are: 5'-CCCRTYCATCTGGAAATCTTGGTTC-3' (forward), 5'-GCTRTRATAATGAGAAAGATTTCTGC-3' (reverse) for matK locus and 5'-ATGTCACCACAAACAGAGACTAAAGC-3' (forward), 5'- GTAAAATCAAGTCCACCRCG -3' (reverse) for rbcL locus..

Two products of PCR amplification (matK and rbcL) were obtained from each plant sample by using the primers mentioned above.

The reaction mix on one sample contained: 7.1ul H_2O, 10ul PCR buffer, forward and reverse primer (10mM) each 1ul, 0.2ul Phire polymerase and 0.5 ul of DNA sample diluted in a "dilution buffer".

PCR thermal profile: 98°C (5 min), 98°C (5 s), 48°C (5 s) (for matK locus) or 54°C (5 s) (for rbcL), 72°C (20 s) The above thermal profile was repeated 40 times and finished by the last elongation stage in 72°C for 1 min and then cooled to 4°C until samples recovery.

The success of the amplification was verified on agarose gel stained with ethidium bromide after electrophoresis. The intensity, length and quality of the products were also verified.

All the resultant products were purified on a "High Pure PCR Product" kit (Roche).

Each purified product (fragment) was sequenced in both directions (forward and reverse) using a DTCS-Quick

Table 1: PCR success summary for loci, species and fragment lengths

Species name	locus rbcL	locus matK	# of native species
Anthoxanthum alpinum	+(650bp)	No amplification	2 of 3
Anthoxanthum odoratum	+(650bp)	No amplification	
Empetrum nigrum	+(650bp)	+(850bp)	2 of 2
Empetrum hermaphroditum	+(650bp)	+(850bp)	
Vaccinium Vitis-ideae	+ (650bp)	+(850bp)	4 of 4
Vaccinium myrtillus	+(650bp)	+(850bp)	
Vaccinium gaultherioides	+(650bp)	+(850bp)	
Vaccinium uliginosum	+(650bp)	+(850bp)	
Rumex acetosa	+(650bp)	+(800bp)	2 of 3*
Rumex alpestris	+(650bp)	+(800bp)	
Symphytum officinale	+(650bp)	+(800bp)	2 of 4
Symphytum tuberosum	+(650bp)	+(800bp)	
Dryopteris expansa	+(650bp)	No amplification	5 of 8
Dryopteris carthusiana	+(650bp)	No amplification	
Dryopteris filix-mas	+(650bp)	No amplification	
Dryopteris borerri	+(650bp)	No amplification	

*subgenus *Acetosa*

Unauthenticated
Download Date | 10/21/17 1:28 AM

Start Kit (Beckman&Coulter). Sequencing was carried out on a GenomeLab GeXP sequencer in compliance with the manufacturer's instructions.

The chromatograms obtained after sequencing were reviewed for signal quality and aligned together. The aligned consensus sequences were used to make a summary sequence comparison between the studied species. Aligned summary was made in BioEdit 7.1.3.0 and a ClustalW software tool included in Bioedit [3].

The dendrograms were computed using MEGA 6.06 [4] software with aligned sequences and option "Construct Neighbor-Joining tree..." with bootstrap option set on 500 repetitions.

3 Results and Discussion

Direct PCR method and primers (details above) used in this research work, made possible for selective amplification from all 55 (100% of the samples) analyzed samples in case of rbcL locus. In case of the matK primers 32 PCR products were obtained (64% of the samples) because in the *Anthoxanthum* and *Dryopteris* species group it was impossible to acquire a good quality PCR product of the matK locus. It confirms that longer DNA fragments are more difficult to amplify and it could be problematic to make successful amplification for old herbarium samples.

In summary, 87 good quality PCR products were proper for sequencing (Table 1). The length of the rbcL fragments was 650bp and for matK locus it was 850bp.

After sequencing, 174 good quality chromatograms were obtained ready for further analysis.

3.1 *Vaccinium* species group

Three out of four selected species are very well defined and easily recognizable both in their vegetative and generative states. Contrary to this, narrowly treated species i.e. tetraploid *Vaccinium uliginosum* s.stricto. (2n = 48) and diploid *Vaccinium gaultherioides* (2n = 24) are very difficult to distinguish based on their morphological features. For certain identification kariological studies are needed. *Vaccinium uliginosum* is a lowland species and *Vaccinium gaultherioides* is a typical arctic – alpine plant. In Central Europe including Poland their ranges do not overlap as *Vaccinium uliginosum* is restricted in its distribution to lowland and lower mountain elevations (colline, lower montane and upper montane belts) whereas *V. gaultherioides* is a high mountain (subalpine and alpine) species distributed above the timberline.

This group of species is characterized by a high variability of the sequence in the studied loci and more importantly, there are unique substitutions specific for each of the investigated species (Tab 2 and Figure 1).

DNA barcoding should be very useful in this species group.

Table 2: List of differences between the sequences of locus matK in the Vaccinium species group

Species name	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
V.vitis-idaea	C	C	C	A	C	A	T	G	C	T	C	C
V.myrtillus	C	C	C	A	A	G	C	G	A	C	G	C
V.gaultherioides	T	T	C	C	A	G	C	G	C	C	G	T
V.uliginosum	T	T	T	A	T	G	C	A	C	C	G	T

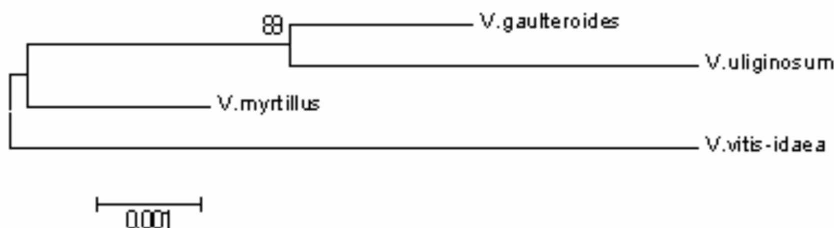

Figure 1. Genetic distances in matK locus in *Vaccinium* genus (NJ tree, bootstrap 500x).

Unauthenticated
Download Date | 10/21/17 1:28 AM

3.2 Locus matK in the *Vaccinium* species group

The sequences obtained in this study revealed a 99% similarity to the sequences of these loci in the genus *Vaccinium* published in the NCBI database.

Of all juxtaposed sequences of the matK locus of the genus *Vaccinium* we have noticed 12 single nucleotide substitutions (Table 2) which differentiate the species.

The first subgroup containing *V.vitis-idaea* and *V.myrtillus* is characterized by the presence of a "C" substitution in M1, M2, M3 and M12 positions of the sequence and by an "A" nucleotide in the M4 place.

The second subgroup combining V.*gaultherioides* and *V.uliginosum* in the M1, M2 and M12 place of the locus sequence contains "T". Each of the studied species is characterized by a unique substitution in the sequence of the matK locus and can be clearly identified based only on this locus sequence. *V.vitis-idaea* contains 5 unique substitutions in the M5, M6, M7, M10 and M11 places of the tested sequence. *V.myrtillus* species is unique only in the M9 position.

V.gaultherioides can be identified based on one substitution in the M4 place and *V.uliginosum* can be distinguished thanks to three substitutions in positions M3,M5 and M8 of the studied sequence.

Based on above results, the population 3576 (the gene bank accession number KF163416.1) from Kevo (Finland) marked during the collection as *V.uliginosum* should be considered as *V.gaulterioides*.

The results seem to confirm the most distinct position of *V.vitis-idaea* (Figure 1.) sometimes treated as a separated genus or subgenus [5,6].

Our results help modify incorrect information concerning the distribution of *V.uliginosum* and *V.gaulterioides* in Poland (Mirek and Combik manuscript in preparation).

The species group *V.vitis-idaea/myrtillus* can be also identified without making expensive sequencing by using Eam1104I or EarI enzyme which detects the CTCTTCN/ sequence motive characteristic for both species. However, the enzyme makes the matK product only 30 bp shorter and as a result cleavage detection may be difficult.

3.3 Loci rbcL in *Vaccinium* species group

The nucleotide sequence of the rbcL locus contains three one-nucleotide substitution showing variability between the species (Table 3).

Detected differences allow for the identification of *V.vitis-idaea* and *V.myrtillus* separately and distinguishing them from the other two species *V.galutherioides* and *V.uliginosum* which have an identical **rbcL** locus sequence. In case of this locus *V.myrtillus* is the most distinct in the species group as it is characterized by 2 unique substitutions instead of only one for *V.vitis-idaea*. (Figure 2).

It is possible to use AluI restriction enzyme for the identification of the *V.vitis-idaea* species. This enzyme recognizes the AGCT sequence motif and cuts the PCR product into two fragments of 370 and 160 bp, respectively. Cleavage detection can be easily performed on agarose gel electrophoresis.

3.4 *Symphytum* species group

The two species used in this study are very well defined and they differ as regards many generative and vegetative features.

Table 3: List of differences between the sequences of the locus rbcL in the *Vaccinium* species group

Species name	M1	M2	M3
V.vitis-idaea	C	T	G
V.myrtillus	A	C	A
V.gaultherioides	C	C	G
V.uliginosum	C	C	G

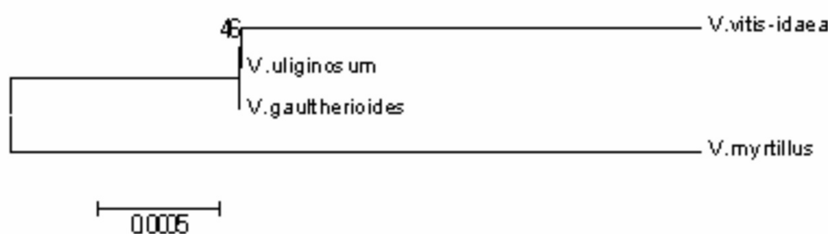

Figure 2. Genetic distances in rbcL locus in *Vaccinium* (NJ tree, bootstrap 500x).

Unauthenticated
Download Date | 10/21/17 1:28 AM

In spite of very significant differences between these species, the sequences of locus matK, believed to be very useful in distinguishing between taxa at the species level, showed no differentiation within the tested samples.

Also, in the nucleotide sequence of rbcL only a very slight difference was found between the tested *Symphytum* species. We found only one substitution occurring in the 385-site sequence. The *Symphytum officinale* species is characterized by the presence of an "A" nucleotide at this point while the *S. tuberosum* sample has a "C" nucleotide. As a result, this locus can be used to distinguish these species from each other.

3.5 *Anthoxanthum* species group

The two species selected for this group represent very closely related taxa which differ very slightly in the indumentum of the *palea* and the *lemma* as well as in their vegetative shoots. For this reason they are treated as very low rank in the Flora of Europe. The author [7] mentions only that among widely treated *A. odoratum* L. diploids are sometime distinguished as *A. alpinum* A. & D Love but according to him it "seems impossible to recognize even subspecies within *A. odoratum*" [p. l.] (Tutin l.c.)

Despite his comment most European floras distinguish the diploid *A. alpinum* 2n = 10 from the tetraploid *A.odoratum* as well defined species with different general distribution and habitats. *Anthoxanthum odoratum* is a lowland species penetrating only into lower mountain elevations; *A. alpinum* is a typical arctic-alpine species occurring in Poland in Sudety Mountains and Carpathian Mountains above 1,200m, i.e. from the upper montane belt up to the alpine belt. In the upper forest belt altitude, the ranges of the species overlap but no intermediates have been found and no evidence of their hybridization in this belt is known [8,9,10].

The analysed samples of *Anthoxanthum* contain only one difference in the rbcL sequence. Unfortunately this one "A" nucleotide substitution occurred in only two samples of *A. odoratum*. Other samples of *A. odoratum* and all the samples of *A.alpinum* contained a "G" nucleotide. This may indicate a variation inside the *A.odoratum* species and does not allow for the use of the rbcL loci to identify the species without a broader analysis of the population.

3.6 *Empetrum* species group

Two closely related species representing *Empetrum nigrum* s.l. of various ploidy levels, general distribution and habitats have been selected for the comparison. *E. nigrum* is a lowland boreal species with a diploid chromosome number (2n = 26) widely distributed in heaths, moors and coniferous forests of Northern Hemisphere[11]. In mountains, the species penetrates only into the lower (sporadically also into upper) montane belt. On the other hand, the tetraploid *E. hermaphroditum* (2n = 52) is a typical arctic-alpine species quite common both in arctic and northern boreal zones and in alpine mountains where it occupies high-mountains heaths above the timberline. In Central Europe, their ranges do not overlap but in some mountains their localities are close to each other. The only conspicuous differences between two species can be observed in the flowering stage (dioecious *E. nigrum* and normally hermaphrodite *E. hermaphroditum*). Hybrids are known as extremely rare both in Far North and Central European mountains and can be found only in localities where two parental species occur together [12,11]. There is some discussion concerning the rank of these two taxa oscillating between varieties and separate species. In Flora Europea (volume 3), D.A. Webb treats them in a specific rank, however in many other cases analogical taxa are treated as separate microspecies.

In our study no difference in the matK and rbcL loci sequence between the tested species of *Empetrum* were found. This can suggest that in such cases barcoding standard loci are not useful or they confirm a lower than specific status of the taxa.

3.7 *Rumex* species group

Only two closely related species were selected for comparison. These are *R. acetosa*, a lowland species going up to the upper part of the lower montane belt in the mountains and the subalpine-alpine *R. arifolius (R.alpestris*) occurring mainly from the upper forest belt to the alpine belt but descending into the lower part of the lower montane belt. Both species, which are diploids (2n = 14), overlap in their altitudinal distribution from 600 to 12,500 m a.s.l. [13]. Differences between these two taxa are not very distinct but there exist several traits which help distinguish between them. In the wide zone, where these two species occur together, very frequent intermediates are observed, most probably of hybrid origin. The specific status of *R.acetosa* and *R. arifolius* is usually not questioned and we found no difference in the matK and rbcL loci sequence between the tested species. It would again suggest that both markers (loci) are not universal enough or the taxonomic rank of these two taxa requires further discussion. In fact, in some regional floras, particularly older ones, they are distinguished at the subspecies level.

Unauthenticated
Download Date | 10/21/17 1:28 AM

3.8 *Dryopteris* species group

Two very distinctive but critical groups of species were selected. The first is the *Dryopteris carthusiana* group with three different species: two allotetraploids *D. cathusiana* (2n = 164) and *D. dilatata* (2n = 164) and one diploid *D. expansa* (2n = 82). The second group is *D. filix-mas* consisting of two species: tetraploid *D. filix-mas* (2n = 164) and di- or triploid *D. borreri* (2n = 82,123). *Dryopteris cambrensis*, recently recognized in our flora as a third representative of the group has not been included in our study. All the species of the *D.carthusiana* group are very easily recognizable by the features of perispore sculpture and the average size of the stomata; the most closely related *D. dilatata* and *D. expansa* differ also in their chromosome numbers [13]. Their morphological features are very distinct and typical forms which overlap in some percentage of the individuals [14]. All the species of this group are widely distributed in mountain areas but their distribution in lowlands differs very much in the frequency and area occupied [15]. Moreover, a high percentage of intermediates of hybrid origin occur in populations were two or three representatives of this group meet together. Analogically, in the *D.filix-mass* group the two species in question differ both in macro morphology, ploidy level and perispore sculpture [16]. They also differ in distribution and habitats.

The differences between the species in the rbcL locus correspond with what we know from their morphology. The most distinct differences (concerning 9 nucleotide substitutions) are those between the two main groups of species (*D. dilatata* and *D. filix-mas*). In *D. dilatata* group, the biggest number of specific substitution (5) was found in *D. expansa* which is astonishing as the species is believed to be very closely related to *D. dilatata*; in our study it is much more similar to *D. carthusiana*. These two species differ only in 3 substitutions. (Table 4)

Very puzzling is the lack of differences in the rbcL locus sequence in *D. filix-mas* and *D. borreri* which differ in morphology more clearly than species of the *D. dilatata* group (See Figure 3.) Unfortunately, in case of the matK locus it was impossible to obtain the PCR product using this sample and the primers described above.

Table 4: List of differences between the locus rbcL sequences in the *Dryopteris* species group

Species name	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13 M14 M15	M16	M17	M18	M19
D. expansa	T	T	C	G	C	A	C	A	G	A	G	C	C	C	C	T	A
D. dilatata	T	T	T	G	T	G	C	A	A	G	A	T	C	A	C	T	A
D. carthusiana	T	T	T	G	C	G	C	A	G	A	A	T	C	A	C	T	A
D. flilix-mas	C	C	T	A	C	G	T	G	G	G	G	T	T	A	T	C	G
D. borreri	C	C	T	A	C	G	T	G	G	G	G	T	T	A	T	C	G

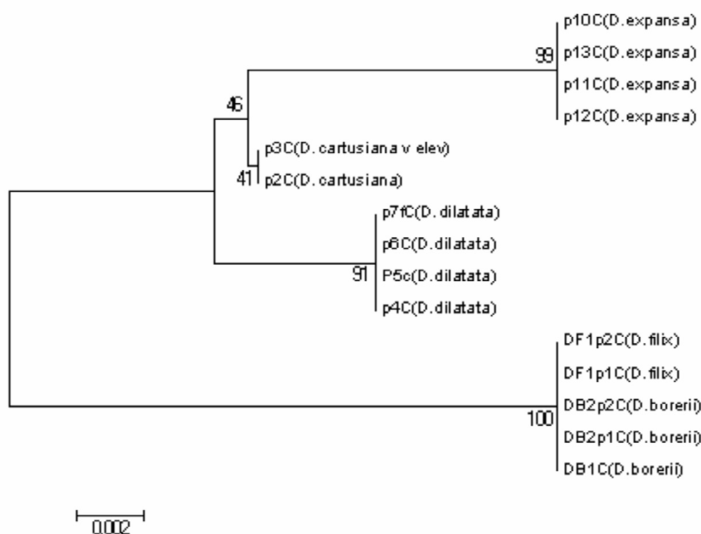

Figure 3. Neighbour-Joining dendrogram showing relationships in the *Dryopteris* species group

Unauthenticated
Download Date | 10/21/17 1:28 AM

4 Summary and Discussion

The results of this study show that it is possible to use DNA barcoding as an additional tool for identifying species for many taxonomical groups of vascular plants. The loci selected as a standard in DNA barcoding of flowering plants and Pteridophytes appeared to be very useful for the identification of well defined species and micro species in some of the selected groups. In this last case they make it possible to distinguish very well between a pair of di- and tetraploids in the *Vaccinium uliginosum* group *(V. uliginosum s.str*. and *V. gaultherioides*) but they do not differentiate between another such pair of species in the groups of *Anthoxanthum odoratum*, *Empetrum nigrum* or Rumex *acetosa*. In such cases one needs to look for other markers and/or other molecular techniques. In case of *Symphytum* genus only rbcL locus is useful.

Barcoding in Dryopteris genus works well for rbcL but matK locus due to problem with PCR amplification was not evaluated. (Table 5)

Out of 17 species selected for this study barcoding helps identify 11 of them, i.e. 64% of the total what is close to result obtained by other researchers [17]. However species selection for this study was made concerning good evaluation of DNA barcoding usability with minimal cost, what can have big influence on evaluation results. Making

Table 5: Summary list of barcoding usefulness.

Generic name	Species name	Sequence of rbcL	Sequence of matK
Vaccinium	*myrtillus*	useful	useful
	vitis-idaea	useful	useful
	uliginosum	Identical to V.gaultherioides/ useful	useful
	gaultheroides	Identical to V.uliginosum useful	useful
Empetrum	*nigrum*	Non useful	Non useful
	hermaphroditum	Non useful	Non useful
Anthoxanthum	*alpinum*	Non useful	No data
	odoratum	Intraspecific variability	No data
Rumex	*acetosa*	Non useful	Non useful
	alpestris	Non useful	Non useful
Symphytum	*officinale*	useful	Non useful
	tuberosum	useful	Non useful
Dryopteris	*expansa*	useful	No data
	dilatata	useful	No data
	carthusiana	useful	No data
	filix-mas	useful	No data
	borreri	useful	No data

Table 6: Detailed information about sample collection site and accesion number In NCBI genebank

Species name	Population number	rbcL accession number	matK accession number	location
Vaccinium myrtillus	3773	KF163410.1	KF163391.1	Poland, Babia Góra, Akademicka Perć 1270 m a.s.l.
	3788	KF163411.1	KF163390.1	Poland, Mały Śnieżny Kocioł 1450 m a.s.l.

Unauthenticated
Download Date | 10/21/17 1:28 AM

continued **Table 6:** Detailed information about sample collection site and accesion number In NCBI genebank

Species name	Population number	rbcL accession number	matK accession number	location
Vaccinium uliginosum	3576	KF163416.1	KF163394.1	Finland, Kevo 105 m a.s.l.
	3809	KF163417.1	KF163395.1	Poland, Tatras, Wyżnia Pańszczycka Młaka 1300 m a.s.l.
Vaccinium gaultherioides	3743	KF163415.1	KF163393.1	Poland, Tatras, Długi Upłaz 1780 m a.s.l.
	~~3576~~	~~KF163416.1~~	~~KF163394.1~~	Finland, Kevo 105 m a.s.l.
	3752	KF163414.1	KF163392.1	Poland, Tatras, Slopes of Ornak
Empetrum	2788	None	KF522687.1	Norway, square W43-3
Empetrum hermaphroditum	3778	None	KF522690.1	Poland, Babia Góra, Akademicka Perć 1650 m a.s.l.
	3340	KF522682.1	KF522688.1	Poland, Tatras, Dubrawiska 1650 m a.s.l.
	1087	KF522681.1	KF522689.1	Scotland, Ben Lawers, Lochan nan Cat. 860 m a.s.l.
Empetrum nigrum	3058	KF522683.1	KF522685.1	Romania, G. Marmaroskie Bardau, 1850 m a.s.l.
	1090	None	None	Poland, Łeba moorland
	1139	KF522684.1	KF522686.1	Poland, Tatras, Goryczkowa Czuba 1780 m a.s.l.
	1231	None	None	Poland, Tatras, Suchy Wierch,1520 m a.s.l.
Vaccinium vitis-idaea	3777	KF163413.1	KF163389.1	Poland, Babia Góra, Akademicka Perć 1650 m a.s.l.
	3804	KF163412.1	KF163388.1	Poland, Karkonosze, Wielki Śnieżny Kocioł 1270 m a.s.l.
Anthoxanthum alpinum	3715	KF522676.1	None	Slovakia, Dolina Litworowa 1838 m a.s.l.
	3880	KF522673.1	None	Austria, Sauaple,Guttaringer Alm 2024 m a.s.l.
	3771	KF522674.1	None	Poland, massif Śnieżka position no 3 1300 m a.s.l.
	3737	KF522675.1	None	Poland, Ornak 1850 m a.s.l.
Anthoxanthum odoratum	3775	KF522677.1	None	Poland, Babia Góra-Zawoja Markowa 850 m a.s.l.
	3706	KF522680.1	None	Poland, Bieszczady, Wetlina 670 m a.s.l.
	3761	KF522679.1	None	Poland, Tatras, Molkówka
	1336	KF522678.1		Poland, Turtul near Suwałki 220 m a.s.l.
Rumex alpestris	258	KF293398.1	KF509938.1	Poland, Tatras, Czarny Staw 1581 m a.s.l.
	342	KF293397.1	KF509936.1	Poland, Bieszczady Wołosate 780 m a.s.l.
	343	KF293396.1	KF509935.1	Poland, Bieszczady, at the entrance of the trail leading to Połonina Wetlińska1080 m a.s.l.
	3845	KF293399.1	KF509937.1	Poland, Tatras, Gąsienicowa Valley

Unauthenticated
Download Date | 10/21/17 1:28 AM

continued Table 6: Detailed information about sample collection site and accesion number In NCBI genebank

Species name	Population number	rbcL accession number	matK accession number	location
Rumex acetosa	260	KF293395.1	KF509934.1	Poland, Wołosate, 770 m a.s.l.
	1126	KF293394.1	KF509933.1	Poland, Kraków, Podgórki Tynieckie, 207 m a.s.l.
	1573	KF293393.1	KF509932.1	Norway, 68º46’31.8” N 24º24’39.8” E, 805 m a.s.l.
	3247	KF509931.1	KF293392.1	Romania, Ciucas, Valea Berii 1090 m a.s.l.
Symphytum officinale	339	KF170561.1	KF170553.1	Poland, Elbląg Canal, Buczyniec
	940	KF170560.1	KF170552.1	Poland, Bieszczady, Izab??
	338	KF170563.1	KF170554.1	Poland, Moszna near Opole
	941	KF170562.1	KF170555.1	Poland, Pieniężno roadside
Symphytum tuberosum	427	KF170559.1	KF170550.1	Poland, LubońWielki over Rabka 1000 m a.s.l.
	436	KF170557.1	KF170549.1	Poland, Połonina Wetlińska 1160 m a.s.l.
	694	KF170556.1	KF170548.1	Romania, Retezat, 830 m a.s.l.
	435	KF170558.1	KF170551.1	Poland, Załuż, Sobień 350 m a.s.l.
Dryopteris expansa	p10	KF539808.1	none	Królowe Rówienki (VIII 2012)
	p13	KF539809.1	none	Królowe Rówienki (VIII 2012)
	p12	KF539806.1	none	Królowe Rówienki (VIII 2012)
Dryopteris dilatata	p7	KF539810.1	none	Królowe Rówienki (VIII 2012)
	p6	KF539811.1	none	Królowe Rówienki (VIII 2012)
	p5	KF539812.1	none	Królowe Rówienki (VIII 2012)
	p4	KF539813.1	none	Trzydniowiański Wierch 950 m a.s.l.
Dryopteris carthusiana	p3	KF539814.1	none	Potok 3 (September 2012)
	p2	KF539815.1	none	(IX 2012) position no 2
Dryopteris filix-mas	DF1	KF539817.1	none	Tatry, position 3 (August 2011)
Dryopteris borreri	DB2	KF539819.1	none	Pilsko- Hala Miziowa (VIII August 2011)
Dryopteris borreri	DB1	KF539820.1	none	Dolina Kościeliska (August 2011)

Unauthenticated
Download Date | 10/21/17 1:28 AM

a reference database containing sequences (barcodes) identifying the species of a certain area is of much cognitive and practical value. However, this requires the prior creation of a reference database. Producing such a tool will require significant funds to finance the sequencing of a large number of samples.

In some taxonomical groups, sequences of plastid loci show intraspecific variability. It can be used to make phylogeographical analysis but could pose problems during barcoding in species identification. That is the case in the *Anthoxanthum* species group. It may be that in those groups wider sampling can result in some potentially interesting phylogeographycal information.

Acknowledgements: This work was funded from N N303 801 440 grant and from grant "Młodzi Naukowcy" (Young Scientists, 2012).

Conflict of interest: Dr Combik declares nothing to disclose.

References

[1] Hollingsworth P., Wilkinson M., A DNA barcode for land plants, Proc. Nat. Acad. Sci. USA, 2009, 106(31): 12794-12797
[2] Hollingsworth P.M., Graham S.W., Little D.P., Choosing and Using a Plant DNA Barcode, PLoS ONE, 2011, 6(5): e19254, doi:10.1371/journal.pone.0019254
[3] Hall, T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucl. Acids. Symp. Ser,1999, 41, 95-98
[4] Tamura K., Stecher G., Peterson D., Filipski A., Kumar S., MEGA6: Molecular Evolutionary Genetics Analysis version 6.0, Mol. Biol. Evol., 2013, 30, 2725-2729.
[5] Garkava-Gustavsson L., Persson H. A., Nybom H., Rumpunen K., Gustavsson B.A., Bartish I.V., RAPD-based analysis of genetic diversity and selection of lingonberry (*Vaccinium vitis-idaea* L.) material for ex situ conservation, Genet. Resour. Crop Ev., 2005, 52, 723–735
[6] Debnath S.C., Inter simple sequence repeat (ISSR) to assess genetic diversity within a collection of wild lingonberry (*Vaccinium vitis-idaea* L.) clones, Can. J. Plant Sci., 2007, 87, 337–344
[7] Tutin T.G., Heywood V.H., Burges N.A., Valentine D. H., Walters S.M., Webb D.A., Flora Europaea, Cambridge University Press, Cambridge, 1964–1980, Volumes 1–5
[8] Filipová L., Krahulec F., The transition zone between *Anthoxanthum alpinum* and *A. odoratum* in the Krkonoše Mts, Preslia, 2006, 78, 317–330
[9] Flegrová M., Krahulec F., *Anthoxanthum odoratum* and *A. alpinum*: life history parameters at two different altitudes, Folia Geobot., 1999, 34, 19-31
[10] Drapikowska M., Variability of *Anthoxanthum* species in Poland in relation to geographical-historical and environmental conditions: morphological and anatomical variation, Biodiv. Res. Conserv., 2013, 30, 3-61
[11] Suda J., Malcová R., Abazid D., Banaš M., Procházka F., Šída O., Štech M., Cytotype distribution in *Empetrum* (*Ericaceae*) at various spatial scales in the Czech Republic. Folia Geobotanica, 2004, 39: 161-171
[12] Suda J., New DNA ploidy level in *Empetrum* (*Empetraceae*) revealed by flow cytometry, Ann. Bot. Fennici, 2002, 39, 133-141.
[13] Löve Ä., Löve D., Chromosome numbers of northern plant species, Icel. Univ. Inst. Appl. Sci., Dep. Agric. Rep, 1948, Ser. B, 3, 1-131.
[14] Szczęśniak E., Tlałka D., Rostański A., Key to identification and descriptions of species of Buckler-ferns (*Dryopteris* Adans.) occurring in Poland. - In: Szczęśniak E., Gola E., Genus *Dryopteris* Adans. in Poland, Polish Botanical Society & Institute of Plant Biology, University of Wrocław, 2009, p. 5-34
[15] Piękoś-Mirkowa H., The *Dryopteris dilatata* complex in the Soviet Far East, Bot. Helv, 1987, 97, 167-177.
[16] Piękoś-Mirkowa H., Dryopteris affinis(Lowe) Fraser-Jenkins – nowy gatunek we florze Polski Dryopterisaffinis (Lowe) Fraser-Jenkins: a new species in the flora of Poland, Fragm. Fl. Geobot, 1981, 27, 359–370
[17] Saarela J.M., Sokoloff P.C., Gillespie L.J., Consaul L.L., Bull R.D., DNA Barcoding the Canadian Arctic Flora: Core Plastid Barcodes (rbcL + matK) for 490 Vascular Plant Species, PLoS ONE, 2013, 8(10), e77982. doi:10.1371/journal.pone.0077982

Unauthenticated
Download Date | 10/21/17 1:28 AM