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SHORT COMMUNICATION

Effort to reconstruct past population history in the fern *Blechnum spicant*

Helena Korpelainen · Maria Pietiläinen

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Abstract Our aim was to evaluate the potential of existing herbarium collections in reconstructing the past population history of the rediscovered clone of the regionally extinct *Blechnum spicant* by comparing it with herbarium material from previous sites of occurrences in Finland and elsewhere in northern Europe, as well as to reveal the genetic and geographic relationship among the samples. We detected a total of nine polymorphic sites within the three sequenced regions, two SCAR markers developed now for *B. spicant* and the chloroplast *trnL-trnF* spacer, totalling 763 bp. Despite low variability, the phylogeographic analysis revealed the presence of some geographic pattern among the samples, of which all except one represented herbarium samples of up to 100 years of age. The rediscovered clone of *B. spicant* proved to represent the prevalent genotype occurring in northern Europe. The studied sequences were neutral in terms of evolution. It is apparent that existing herbarium collections are useful resources for a range of evolutionary and population studies.

Keywords *Blechnum spicant* · Blechnaceae · Herbarium specimens · Phylogeographic analysis · Population history · Sequence diversity

Introduction

Phylogeography allows the inference of evolutionary processes that have shaped the current distribution of lineages across the landscape by revealing the relationships between genetic characteristics and geographic occurrences. Such studies have been conducted in a number of organisms (e.g., Su et al. 2004; Grivet et al. 2006; Schönswetter et al. 2006), but ordinarily are based on newly collected specimens and not on existing herbarium resources. Herbaria are important and frequently used sources for studying systematic questions, including through molecular biological approaches. There are previous reports that 100- to 200-year-old herbarium samples have been used successfully in DNA studies (Fay and Cowan 2001; Larsson and Jacobsson 2004). Current interest is to expand the use of herbarium material to include a wider range of evolutionary and population studies. For instance, Kovarik et al. (2005) have examined concerted evolution of rRNA genes in species belonging to the genus *Tragopogon* and in their alloploids that have formed during the last 80 years, with the help of herbarium specimens representing the early occurrence of alloploidy within the genus.

The fern *Blechnum spicant* (L.) Roth (Blechnaceae) is a widely distributed, long-lived, perennial fern. It reproduces both sexually and vegetatively. *Blechnum spicant* is native to Europe, western North America and northeastern Asia, but it is now considered threatened in parts of Europe. In Central Europe and in the western parts of North Europe, primarily in areas with an oceanic climate, *B. spicant* is still relatively common in moist, coniferous forests. In Finland, it previously occurred as a rare species in southern and most northern parts of the country until it was classified as regionally extinct in 1990, the main causes for the disappearance being forest management practices and peatland drainage (Rassi et al. 2001). *Blechnum spicant* has also been lost from the Estonian flora (Külvik 1998). However, a small stand of *B. spicant*, apparently a single

H. Korpelainen (✉) · M. Pietiläinen
Department of Applied Biology, University of Helsinki,
P.O. Box 27, 00014 Helsinki, Finland
e-mail: helena.korpelainen@helsinki.fi

clone, was found in eastern Finland in 1998 in a region where it had never been previously discovered (Kaukonen 1998).

In the present study, we aimed to evaluate the potential of existing herbarium collections for reconstructing the past population history of plants, and specifically to analyse the genetic characteristics of the rediscovered *B. spicant* clone by comparing it with herbarium material obtained from previous sites of occurrences in Finland and elsewhere in northern Europe during the past 100 years, as well as to reveal the genetic and geographic relationship among samples of *B. spicant*.

Materials and methods

Plant material

Sample 1 of *B. spicant* was collected in 1999 from eastern Finland in the county of Puolanka, where it had been observed in 1998 and a specimen had been collected and placed at the Botanical Museum of Oulu. This was the first observation of *B. spicant* in Finland after it was classified as extinct in 1990 (Rassi et al. 2001). To elucidate the history of this sample, we obtained leaf samples of all 17 *B. spicant* specimens available in the Helsinki Herbarium, University of Helsinki. Two samples, which resulted in DNA of inadequate quality, were excluded from the study. The 15 samples examined originated from Finland, Sweden, Norway, Denmark and Iceland and had been collected between 1902 and 1990 (Table 1; Fig. 1). All leaf samples, including those excluded from the study, were green and appeared of even quality. As an insect control method, the Herbarium used carbon disulphide treatments for several decades until late 1970s, and freezing thereafter. The same specimens may have been exposed to treatments several times.

DNA marker development and sequencing

We extracted total DNA from leaf material using DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions except for allowing the plant material to incubate for 30 min on ice instead of the suggested 5 min at the precipitation step in order to improve the yield of DNA. The analysis involved the PCR amplification and sequencing of three DNA regions, one within maternally inherited cpDNA, the non-coding chloroplast *trnL-trnF* spacer, and two within sequence-characterized amplified regions (SCAR) markers developed for *B. spicant*. The development of the SCAR markers involved the amplification of genomic DNA (sample 1) with inter simple sequence repeats (ISSR) primers $(TC)_8A$ and $(CT)_8G$ [one primer per reaction, both primers belong to the primer set used when screening for highly variable genome regions (Korpelainen et al. 2007)] in a volume of 20 µl (reaction conditions as in Korpelainen et al. 2005), followed by electrophoretic separation of the amplified fragments, and excision and purification of clear fragments with QIAquick Gel Extraction Kit (QIAGEN). The purified DNA was concentrated to 3–5 µl and cloned using the TOPO TA Cloning kit (Invitrogen). The amounts of reagents used were reduced by 50% in proportion to the amounts recommended by the manufacturer. Positive clones were selected from the plates and transferred into 50 µl of ddH_2O. DNA amplification was conducted in 20-µl reaction volumes containing 4 µl clone solution, 0.5 units of Phusion High-Fidelity DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 µl 10 mM dNTP mix, and 1 µl each of primers M13-f and M13-r (at a concentration of 5 µM) included in the cloning kit. The thermocyler was programmed for 30 s denaturation at 98°C, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and elongation at 72°C for 50 s. An additional 8-min elongation followed the last cycle. The amplification products were separated electrophoretically, excised from the gel and purified.

The following sequencing was conducted using the M13-f primer. Primer pairs were then designed for two cloned and sequenced fragments to allow the amplification and sequencing of the ISSR fragments (now called SCARs) with the PCR primers, and the detection of sequence polymorphisms within the SCARs. The primer sequences used for the amplification and sequence analysis of the SCARs *BS2a* and *BS3a* were as follows: BS2a-f 5′-AGA GAGAGAGTTGATTGACCTAC-3′ and BS2a-r 5′-GAGA GAGAGAGTCTAACTACATAAC-3′, and BS3a-f 5′-GA GATCAGGGGGCTCTTAATTATA-3′ and BS3a-r 5′-AG TACCTCAATAGAGCACTCTTAC-3′. The 20-µl reaction volumes contained about 10–20 ng of genomic DNA, 1.2 units of DyNAzyme II DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 µl 10 mM dNTP mix, and 1 µl of both 5 µM primers. The thermocyler was programmed for denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and elongation at 72°C for 60 s. An additional 8-min elongation followed the last cycle. The chloroplast DNA was amplified with primers designed for the *trnL-trnF* spacer (primers trnL-f 5′-GGTTCAAGTCCCTCTATC CC-3′ and trnF-r 5′-ATTTGAACTGGTGACACGAG-3′; Taberlet et al. 1991). The reaction conditions for the amplification of the *trnL-trnF* spacer were similar to those of the SCAR markers except for the annealing temperature of 50°C. The amplification products were separated electrophoretically, excised from the gel, purified and sequenced using the forward primers.

Table 1 Collecting sites of *Blechnum spicant*, and voucher and GenBank accession numbers

Sample	Location	Collecting year	Coordinates	Voucher	*SCAR-BS2a*	*SCAR-BS3a*	*trnL-trnF* spacer
1	Puolanka, Finland	1998	64°07′N 29°31′E	171471	EF407537	EF407521	EF427625
2	Porvoo, Finland	1907	60°24′N 25°40′E	092313	EF407538	EF407522	EF427626
3	Saltvik, Åland, Finland	1950	60°17′N 20°03′E	092303	EF407539	EF407523	EF427627
4	Geta, Åland, Finland	1918	60°23′N 19°51′E	092307	EF407540	EF407524	EF427628
5	Gotland, Sweden	1927	57°30′N 16°33′E	1127627	EF407541	EF407525	EF427629
6	Halland, Sweden	1902	57°05′N 11°58′E	1127030	EF407542	EF407526	EF427630
7	Halland, Sweden	1977	57°05′N 11°58′E	1151566	EF407543	EF407527	EF427631
8	Jylland, Denmark	1968	57°07′N 08°39′E	1127654	EF407544	EF407528	EF427632
9	Gothenburg, Sweden	1935	57°42′N 11°58′E	1127577	EF407545	EF407529	EF427633
10	Telemark, Norway	1962	59°27′N 08°23′E	1127651	EF407546	EF407530	EF427634
11	Åre, Sweden	1946	63°25′N 13°05′E	1127591	EF407547	EF407531	EF427635
12	Åre, Sweden	1968	63°25′N 13°05′E	1108911	EF407548	EF407532	EF427636
13	Brekkvasselv, Norway	1967	65°02′N 11°56′E	1579154	EF407549	EF407533	EF427637
14	Austvågaoya, Norway	1990	68°16′N 14°30′E	1650372	EF407550	EF407534	EF427638
15	Troms, Norway	1990	68°50′N 14°56′E	1650557	EF407551	EF407535	EF427639
16	Holsdalur, Iceland	1964	65°39′N 23°48′E	1127661	EF407552	EF407536	EF427640

Samples represent herbarium material from the Helsinki Herbarium, except sample 1. The voucher collected from the site of sample 1 is deposited at the Botanical Museum of Oulu (OULU)

Data analysis

We aligned the sequences using Clustal W (Thompson et al. 1994) and later hand-edited them. The sequences of the most informative single region, *SCAR-BS2a*, presumed to represent nuclear DNA, were subjected to a further phylogeographic analysis. In the analysis, the heterozygous character states were considered as being similar to homozygous character states, which lowered the number of informative sites to five. After that, the sequences of five polymorphic sites were subjected to bootstrapping with SEQBOOT 1,000 times, after which the data considered now to be discrete were clustered using the program PARS, which carries out the Wagner parsimony method with multiple states (Kluge and Farris 1969); both programs are from the PHYLIP 3.63 software package (Felsenstein 2004). The program CONSENSE was then used to find the consensus tree, and DRAWGRAM was used to plot an unrooted tree, all programs from the PHYLIP package. We used the software Arlequin 3.01 (Excoffier et al. 2005) when conducting Tajima's test (1989) of selective neutrality across the three sequenced DNA regions totalling 763 bp.

Results and discussion

The lengths of the SCAR regions *BS2a* and *BS3a* equaled 227 and 221 bp, respectively. *BS2a* contained five polymorphic nucleotide sites within the sequence positions 89–99 and two polymorphic sites (either homozygous C or presumably heterozygous A/C) at positions 127 and 129, while *BS3a* contained one polymorphic site at position 186 (Table 2). The length of the sequenced *trnL-trnF* spacer region equaled 315 bp and showed polymorphism only at position 50. Therefore, the SCAR marker *BS2a* was the most variable and informative region among the three regions examined. It also contained a microsatellite repeat region $(CT)_{11}(CA)_{13}$, which, however, did not contain any variation among the sequenced samples. The DNA fragments used for the development of SCAR markers contained TC or CT repeats in both ends due to the ISSR technique used for generating the DNA fragments to be characterized. It has been previously observed that microsatellite-type elements tend to accumulate in certain genomic regions instead of being evenly distributed (Estoup et al. 1999; Altenheim et al. 2002; Korpelainen et al. 2007). ISSR-based methods can also be used as search tools for variable genetic elements, including microsatellites (Korpelainen et al. 2007).

Despite low sequence divergence (Table 2), the cluster analysis conducted for the *SCAR-BS2a* data resulted in quite high bootstrapping percentages (Fig. 2), and the phylogeographic comparison allowed us to determine some connections between the genotypes of the 16 samples of *B. spicant*. Sample group A represents a prevalent genotype, as it consists of nine samples and is widely distributed (Fig. 1). Also, the recently found occurrence of *B. spicant* in eastern Finland (sample 1) is of this genotype, along with the 100-year-old sample from southern Finland

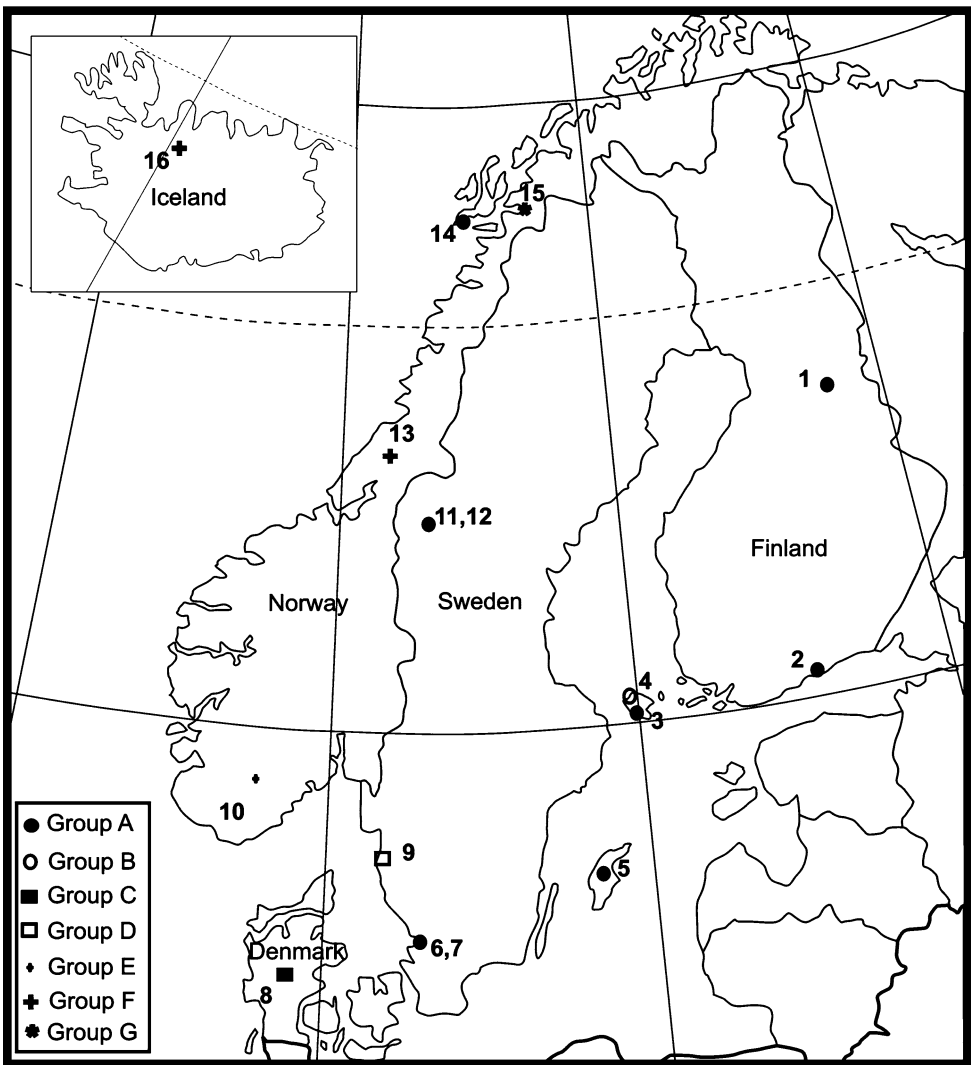

Fig. 1 Collecting sites of *Blechnum spicant*. The samples and sample groups (A–G) are explained in Tables 1 and 2

Table 2 Polymorphic sites (marked in *bold*) in the DNA regions (*SCAR-BS2a*, *SCAR-BS3a* and the *trnL-trnF* spacer, total 763 bp) studied in *Blechnum spicant*

Sample groups	Polymorphic positions			
	SCAR-BS2a (227 bp)		*SCAR-BS3a* (221 bp)	*trnL-trnF* spacer (315 bp)
	89–99	127–129	186	50
A: 1–3, 5–7, 11–12, 14	-C-CTC-**T**CC-	**C**T**C**	**G**	**A**
B: 4	**G**C**G**CTC**GT**CC**G**	**M**T**M**	**G**	**A**
C: 8	-C-CTC-**T**CC-	**M**T**M**	**G**	**G**
D: 9	**T**C**G**CTC**GT**CC**G**	**M**T**M**	**G**	**A**
E: 10	-C**G**CTC-**T**CC-	**M**T**M**	**G**	**G**
F: 13,16	-C**G**CTC-**G**CC-	**M**T**M**	**G**	**A**
G: 15	-C**G**CTC-**G**CC-	**M**T**M**	**T**	**A**

M = A or C

(sample 2) and the almost 60-year-old sample from the Finnish archipelago (sample 3). Sample group C (sample 8) is closely related to group A but has one substitution in the chloroplast *trnL-trnF* intron and is heterozygous for the *SCAR-BS2a* sites 129 and 129. Groups C and E (sample 10) represent southwestern plants, and both samples have a chloroplast genotype different from the prevalent genotype. Groups F (samples 13 and 16) and G (sample 15), the latter group having the only nucleotide difference found in *SCAR-BS3a*, represent distinct northwestern plants. Groups B (sample 4) and D (sample 9) are distinctive genotypes, which differ from each other by one nucleotide only and

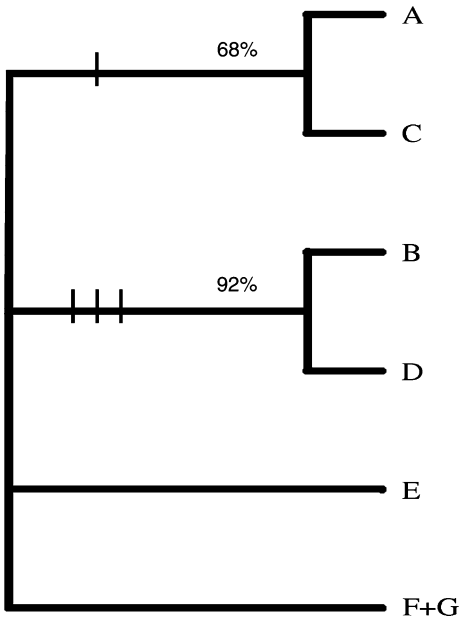

Fig. 2 Cluster analysis of *Blechnum spicant* based on the *SCAR-BS2a* sequence totalling 227 bp. The bootstrap percentages above 50 are marked. Groups A and C were treated as different OTUs, since group C differed from group A in being heterozygous for two sites (see Table 2). Groups F and G were considered identical in this analysis, but they possessed other sequence differences as shown in Table 2. Each *vertical bar* indicates a synapomorphic character (all indels)

are clustered together (Fig. 2). The three loci examined (two nuclear and one plastid) are independent and do not necessarily share the same history, as indicated by the presence of the same rare chloroplast genotype (G instead of A on site 50 in the *trnL-trnF* spacer) in groups C and E, which, however, do not cluster together in the tree constructed based on variability in the nuclear *SCAR-BS2a* sequence.

Tajima's *D* based on the number of polymorphic sites equaled −0.9751. The value is not significantly different from 0, which indicates that the neutral mutation hypothesis can explain the DNA polymorphism present within the three non-coding DNA regions investigated in *B. spicant*. It follows that selection is not responsible for the pattern of molecular variation detected in *B. spicant*.

The used herbarium material was up to 100 years old. Yet, 15 out of the original 17 samples resulted in DNA of adequate quality for further PCR-based sequencing analyses. The two deteriorated samples originated from Finland and had been collected in 1952 and 1963. The reason for the deterioration is unknown. The studied DNA regions were short (221–315 bp), and that may have been a factor contributing to the mostly successful PCR and sequencing. It is also known that the method of drying, not so much the age of the sample, is crucial to the quality of DNA (Jankowiak et al. 2005). Ferns tend to dry very rapidly in the press, consequently, perhaps, retaining better quality DNA than many other plants. In any event, it is apparent that herbarium material has a high potential to be used not only when conducting systematic studies, but also when examining past intraspecific events and when screening and comparing the genetic diversity of a species at present and in the past, including issues related to the conservation of a species at the edge of its range.

In this particular study, although we were able to prove the usability of old samples and to develop new species-specific markers, the limited number of available specimens and the relatively low level and insufficient structuring of variation within the sequenced regions restricted the scope of the analyses and conclusions. Additional field sampling in the present-day populations would allow more definite conclusions of the phylogeographic pattern of *B. spicant*. Regardless, some new phylogeographic knowledge was gained and the rediscovered clone of *B. spicant* appeared to represent the most prevalent genotype in the species.

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