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TECHNICAL NOTE

Ten polymorphic microsatellite markers for *Hieracium* s.s. (Asteraceae)

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Abstract Eight novel microsatellite markers were developed for *Hieracium* s.s. (excluding *Pilosella*) using a modified FIASCO protocol, and two out of nine microsatellites that were originally developed for *Pilosella officinarum* were optimised for *Hieracium* s.s.. All ten microsatellites consistently amplified loci from a wide range of representatives from north and central European species of various sections of the genus. When tested on 19 individuals, nine microsatellites were found to be highly polymorphic with 6–29 alleles, while one microsatellite marker was monomorphic. Cross-amplification showed that six of the ten microsatellites also amplified products in one taxon of *Pilosella*, *P. floribunda* × *officinarum*.

Keywords Microsatellite markers · FIASCO · *Hieracium* · *Pilosella* · Genetic variation · Cross-amplification

Introduction

Hieracium s.s. (excluding *Pilosella*) is probably the most taxon-rich genus in the plant kingdom. Several thousand taxa, of which the overwhelming majority are polyploids with an apomictic mode of reproduction, have been described. The taxonomic treatment of these taxa varies considerably, both between the two major 'schools' dominating in northern and central Europe, respectively, and between older and more recent studies (cf. Schuhwerk 2002; Tyler 2006). The different treatments partly reflect fundamentally different views of the evolutionary processes and the pattern of morphological variation found within the genus, suggesting that a better knowledge of these processes is urgently needed. One way to advance our current understanding of these taxa is by developing specific molecular markers that can provide insights about the degree of hybridization, gene flow and phylogeny of taxa.

Despite the size of the genus, its intriguing variation and major contribution to the vegetation of most of Europe, only a few molecular studies have been undertaken to date. This is because several researchers have found it markedly difficult to find suitable polymorphic marker systems for this genus (Gaskin and Wilson 2007, T. Tyler unpublished data, J. Fehrer pers. comm., Dolores Lledo pers. comm.). In early attempts, Shi et al. (1996) and Stace et al. (1997) used allozymes to study British members of *H.* sect. *Alpina*, and Mráz et al. (2001) used the same technique to study a small group of Carpathian species. These studies showed that some morphologically defined species consisted of single genotypes, whereas others consisted of several genotypes. However, the number of species included in these studies does not allow for general conclusions concerning evolutionary patterns in *Hieracium*, and a pilot study with allozymes in Swedish members of *Hieracium* revealed only very limited variation and serious methodological problems (T. Tyler, unpublished data). More recent attempts to study molecular variation in *Hieracium* have been made by Storchova et al. (2002) and Rich et al. (2007) using RAPD

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and AFLP-markers, respectively. However, while both RAPD and AFLP markers are useful for answering some questions, they suffer several shortcomings, for instance dominance and the significant production of spurious results. Chapman et al. (2004) developed ISSR markers to study introduced and invasive *Hieracium* species in New Zealand, but the utility of their markers has not been tested on any additional material. Mráz et al. (2005) used chloroplast sequences, in combination with other markers, specifically to detect recent hybridization between two species of the genus, but only low levels of variation were detected with that method.

Studies of genetic variation and molecular markers are much more numerous in the closely related genus *Pilosella* (sometimes treated as *Hieracium* subgen. *Pilosella*), where allozymes (Tyler 2005; Bruun et al. 2007), RAPD and ISSR markers (e.g. Chapman and Brown 2001; Houliston and Chapman 2001), as well as microsatellite markers (Zini and Komjanc 2008) and sequence data have been frequently used.

The aim of the present study was to develop novel microsatellite markers as well as to test nine microsatellite markers previously developed for *Pilosella* (Zini and Komjanc 2008) for their applicability in *Hieracium* s.s. Our aim was to develop microsatellite markers for *Hieracium* that will allow investigating the structure of molecular variation among a wide range of north and central European apomictic and polyploid members of this genus, and thus be a crucial step forward to gain a better understanding of the taxonomy and evolution of this genus (cf. Tyler 2006). In addition, knowledge about the variation of this genus in more detail will also have implications for conservation, as the large number of taxa described in this genus, of which many are local endemics, are currently threatened by the ongoing destruction of habitats (cf. Tyler and Bertilsson 2009).

Materials

We used 19 accessions belonging to seven sections of the genus *Hieracium* s.s., and one representative of *Pilosella* (*P. floribunda* × *officinarum*) from among the accessions cultivated in the experimental garden of the Department of Ecology, Lund University, Sweden (taxonomy according to Tyler 2006). All accessions and species used are of known North European origin (collection data available on request), and they represent a wide taxonomic range of the genus that predominates in northern Europe. The ploidy level of the accessions was known from previous studies with flow cytometry (Tyler and Jönsson 2009) and all samples except for the diploid *H. umbellatum* represent apomictic (micro-)species. Vouchers have been deposited in herbarium LD.

DNA extraction

Genomic DNA was extracted from fresh leaves according to a modified protocol based on Storchova et al. (2000) and Doyle and Doyle (1990): extraction buffer, lysis buffer and TE buffer recipes were taken from Storchova et al. (2000), while the wash buffer recipe was taken from Doyle and Doyle (1990). We started with the Storchova protocol, and hence skipped the step were plant tissue is submerged in NaCl/CTAB. Fresh plant tissue was cut into pieces, mixed with 1.0 ml of extraction buffer and grinded in a mortal. All volumes were doubled as compared to the protocol given by Storchova et al. (2000) and this protocol was followed as far as to the chloroform:isoamylalcohol (24:1) step, after which we followed the protocol described by Doyle and Doyle (1990). The pellets were allowed to dry overnight and then dissolved in 50 µl 1× TE buffer.

The extracted DNA was tested by Polymerase Chain Reaction (PCR) with a final volume of 25: 19.0 µl ddH_2O, 2.5 µl 10× polymerase buffer with $MgCl_2$, 0.5 µl (10 mM) dNTP's, 0.2 µl (25 pmol/µl) forward primer, 0.2 µl (25 pmol/µl) reverse primer, 0.6 µl (1 U/µl) Taq polymerase (Applied Biosystems), 2.0 µl (15 ng/µl) template. Conditions were as follows: 94°C 45 s, unique annealing temperature 1 min 15 s, 72°C 1 min 15 s, for 30 cycles (Tzen-Yuh et al. 1998) and products were separated on an agarose gel to check the quality and length of the products. Subsequently, extracted DNA of plants belonging to three accessions, representing the sections *Tridentata*, *Bifida* and *Hieracium*, were selected for the development of an enrichment library, whereas the remaining 16 were used in the testing of the microsatellites only.

Microsatellite isolation and development

The microsatellite enrichment library was constructed according to the 'fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats' (FIASCO) protocol following Zane et al. (2002) as modified by Wellenreuther et al. (2009). In short, each sample (10 µl of DNA, concentration 25–50 ng/µl) was digested in 6.5 µl ddH_2O, 2 µl buffer, 1 µl BSA and 0.5 µl of Fermenta's Sau3AI (10 U/µl). The sample was then ligated with 3.9 µl ddH_2O, 0.5 µl 1× ligation buffer, 0.5 µl (50 µM) Hell adaptors (Metabion) and 0.1 µl (10 U/µl) of T4 ligase. In the following PCR with Hell 1-primer we used: 1.42 µl ddH_2O, 2 µl 10× polymerase buffer, 2 µl (25 mM) $MgCl_2$, 4 µl (1.25 mM) dNTP's, 0.5 µl (100 µM) primer mix Hell 1 (Metabion), 0.08 µl (5U/µl) Taq polymerase (Applied Biosystems), 10 µl of the digested and diluted DNA (diluted 1:10). PCR conditions were as follows: 94°C 30 s, 56°C 1 min, 72°C 1 min, for 20, 24, 28

and 35 cycles. The samples with the most evenly amplified smears were pooled and used for library construction. In the following hybridisation, 100 pmol of five biotinylated probes were used—$(ATT)_6$, $(GAA)_5$, $(AT)_{10}$, $(AC)_7$, and $(AG)_7$. Magnetic beads (Roche Diagnostic GmbH) were used to separate the DNA with incorporated probes from the fragments without probes.

Following capture of DNA fragments with the magnetic beads, a PCR with 9.79 µl ddH_2O, 2 µl 10× polymerase buffer, 2 µl (25 mM) $MgCl_2$, 4 µl (1.25 mM) dNTP's, 0.13 µl (100 µM) primer mix Hell 1 (Metabion), 0.08 µl (5 U/µl) Taq polymerase (Applied Biosystems) was performed. The conditions were the same as in the previous PCR's, for 20, 25 and 35 cycles.

To clone the amplified fragments, the TOPO-TA cloning kit (Invitrogen) was used, following the manufacturer's instruction. 376 colonies were picked manually, suspended in 150 µl ddH_2O and incubated at 999°C for 3 min. A PCR was run on 192 colonies: 1× polymerase buffer (Applied Biosystems), 1.1 mM $MgCl_2$, 125 µM dNTP's, 0.4 µM primer M13 F (5′-TGTAAAACGACGGCCAGT-3′), 0.4 µM) primer M13 R (5′-CAGGAAACAGCTATGACC-3′), 0.5 U Taq polymerase (Applied Biosystems), 2 µl template in a final volume of 25 µl. PCR conditions and number of cycles followed the manufacturer′s instructions of the TOPO-TA cloning kit. 80 of the 192 colonies were selected, and 2 µl of template DNA was used in a sequencing reaction using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) together with the M13 forward primer. The PCR products were sequenced on an ABI PRISM 3130 xl GENETIC ANALYZER (Applied Biosystems/Hitachi).

Of the 80 sequenced fragments, 64 contained microsatellites motifs. These were selected for reverse sequencing with the M13 primer, using the same conditions as above. Consensus sequences were computed from the forward and reverse sequences, using BioEdit Sequence Alignment Editor ver. 7.0.9.0, and the resulting 44 unique consensus sequences were used to design primer pairs using the Primer-3 program (Rozen and Skaletsky, 2000).

Microsatellite testing

The 44 designed microsatellites primer pairs from the enrichment library, as well as the nine microsatellite markers developed for *Pilosella officinarum* by Zini and Komjanc (2008), were tested on ten individuals. The DNA was amplified using the following conditions:1× polymerase buffer (Applied Biosystems), 1,5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM forward primer, 0.2 µM reverse primer, 0.6 U Taq polymerase (Applied Biosystems), 2.0 µl template. PCR conditions were: 94°C 45 s, unique annealing temperature 1 min 15 s, 72°C 1 min 15 s, for 30 cycles (Tzen-Yuh et al. 1998). The products were separated and visualized on agarose gels.

Of the 44 primer pairs developed, 25 produced distinct bands of suitable length. Of the nine primer pairs developed by Zini and Komjanc (2008), four produced discernable bands. The forward primer of all 29 markers was labelled with Cy5, run on an ALF express II (Amersham Pharmacia Biotech) and analyzed with ALFwin Fragment Analyzer 1.00. The ten most promising, eight from the enrichment library and two from the work by Zini and Komjanc (2008), were chosen for a second test on 10 additional individuals to quantify genetic variation.

Results, conclusions and discussion

The present work describes ten microsatellite primers (Table 1) that produce products in most north European members of *Hieracium* s.s. (Table 2). Data for the eight newly developed microsatellite primers and the two developed by Zini and Komjanc (2008) are summarised in Table 1. The results of these primers (tested on 19 accessions of *Hieracium* and one representative of *Pilosella*) are shown in Table 2.

All of the eight newly developed primers amplified almost all of the 20 accessions representing a wide range of North European *Hieracium* and *Pilosella* taxa, and produced strong and distinct bands. In addition, two of the microsatellite primers adopted from Zini and Komjanc (2008) were found to amplify products in *Hieracium* s.s. Each primer/accession combination was only tested once and the occasional failure of some of these combinations (see Table 2) may be due to PCR failure and/or insufficient quality of the DNA extracted. In most of the cases when amplification could not be unambiguously verified (Table 2), weak bands were visible on the gels. Most probably, at least some of these bands represent a PCR product, but since such bands may also be due to contamination or leakage between the wells on the gel, the presence of an amplification product could in these cases not be ascertained by our tests (Table 2). Thus, failure to produce discernable bands does not necessarily mean that the microsatellite for that particular species did not work, but instead indicates that further works needs to be carried out. In addition, improved amplification quality may be obtained if the PCR conditions are further optimized for each particular taxon.

All but one of the microsatellites developed in our study revealed considerable polymorphism within the group of taxa studied; and between 6 and 29 different alleles were found for each microsatellite. Most probably even more alleles may be revealed if additional taxa are analysed, making these markers highly valuable for discriminating between taxa and for studies of the pattern of molecular variation within the genus.

Table 1 Ten microsatellite primer pairs that amplified products in *Hieracium* s.s

Name	Primer sequence (5′-3′)	Ta (°C)	Size (bp)	Motif	Alleles	Alleles/ ind.	GeneBank accession number
1 3_(C)7	F:TCTACATAGCTCGGAAGGAGGT R:GCGGCACATAAACTGCAA	59.4	102–111	(C)7	6	2	GQ915394
2 3_(TG) 3 + 7 st	F:AACCCTAAATCGTATGTGTCTAGTG R:CCTCCTTCCGAGCTATGTAGA	58.2	153–207	(TG)3, (GT)3, (TG)3, (GAA)3, (AG)5, (AG)9, (AG)4, (C)6	29	5	GQ91539
3 A4(H2)_(AC)6	F:CCAGTTTCAACACACATACGAAC R:CATCTCCCGATTTATCAGTGTG	59.5	111–122	(AC)6	6	3	GQ915396
4 A4(H2)_(A)6_(AT)4	F:CATCTTGTCCGGCCATATCT R:TAGTGGTTGTGCCGACACTT	59.6	147–197	(A)6, (AT)4	7	3	GQ915397
5 E3(A1)_(GTT)3	F:ATTTACCGAAGAGGCGATTG R:CCATGTCATCGTCATCATCC	59.5	183–188	(GTT)3	7	2	GQ915398
6 F4(D2)_(CA)7_(CT)3	F:CCAACATGGACCAAGTCAAC R:TTCTAGGACCACTTGGATAAGC	58.2	138–173	(CA)7, (CT)3	15	5	GQ915399
7 F5(H1)_(GT)6	F:TTTCGTGATTTACACCTTTT R:CCGAAAACAATGGGGTTCT	56.2	179–208	(GT)6	22	6	GQ915400
8 G8(F3)_(A)6	F:AGAAAGAATGGGAAGCTGGT R:TTCTTGTGCCAGATGGAGTG	58.8	194–222	(A)6	14	4	GQ915401
9 HP-3B	F:CCCCAAAACTCCCAATACAT R:TCATTGGGACTTCCACAAGTT	62	116–160	(GT)16	19	7	EF081015
10 HP-9	F:TCTCTTCCTTCCATTCTCATTTG R:TCACGTCATGCTCCAATCTC	63	94	(GA)22, (GT)11	1	1	EF081014

For each primer pair, the forward and reverse primer sequence, annealing temperature (Ta), size range of alleles observed (in basepairs), repeat motif, number of alleles observed in the 20 analyzed samples (cf. Table 2), the maximum observed number of alleles per individual and the GeneBank accession number are given. The two last primer pairs were developed by Zini and Komjanc (2008) for *Pilosella officinarum*, whereas the remaining eight have been developed in this study

Table 2 Cross-amplification of the ten microsatellite primer pairs (cf. Table 1) on 19 accessions of species belonging to different sections of *Hieracium* s.s., and one representative of *Pilosella*

Acc. No.	Species	Section	Ploidy	1	2	3	4	5	6	7	8	9	10
1254	*H. caesiiflorum*	Bifida	3x	+	+	+	+	+	+	+	+	−	−
889	*H. itharophyton*	Bifida	4x	+	+	+	+	−	+	+	+	−	−
22	*H. cf. plumbeum*	Bifida	5x	+	+	+	+	+	+	+	+	+	+
143	*H. ravidum*	Bifida	4x	+	+	+	+	+	+	+	+	+	−
553	*H. umbellatum*	Hieracioides	2x	+	+	+	+	+	+	+	+	+	−
1223	*H. caliginosum*	Hieracium	4x	+	+	+	+	+	+	+	+	+	+
1235	*H. hjeltii*	Hieracium	4x	+	+	+	+	+	+	+	+	−	−
54	*H. expallescens*	Hieracium	4x	−	−	−	−	−	−	−	−	−	−
532	*H. norvegicum* agg.	Oreadea	3x	+	+	+	+	+	+	+	+	+	+
448	*H. saxifragum s.l.*	Oreadea	3x	+	+	+	+	+	+	+	+	−	−
149	*H. sabaudum*	Sabauda	4x?	+	+	+	+	+	+	+	+	−	−
11	*H. cruentiferum*	Tridentata	3x	+	+	+	+	+	+	+	+	−	−
498	Unidentified	Tridentata	4x	+	+	+	+	+	+	+	+	−	−
789	Unidentified	Tridentata	4x	−	+	−	−	+	−	−	+	+	+
15	*H. anfractum*	Vulgata	3x	+	+	+	+	+	+	+	+	+	−
106	*H. neopinnatifidum*	Vulgata	3x	+	+	+	+	+	+	+	+	+	+
243	*H. subarctoum*	Vulgata	4x	+	+	+	−	+	+	+	+	+	+
1193	*H. trichelliceps*	Vulgata	3x	+	+	+	+	+	+	+	+	+	+
1304	*H. oblaqueatum*	Vulgata	4x	+	+	+	+	+	+	+	+	+	−
1325	*P. floribunda x officinarum*	–	?	−	−	+	+	−	+	+	+	+	−

A '+' represents the successful amplification of one or more microsatellite alleles whereas '−' denotes that amplification was incomplete or missing

However, it is noteworthy that among the nine primers developed for Italian *Pilosella officinarum* by Zini and Komjanc (2008), only four amplified alleles in any of the species tested in our study and only two of them were found to consistently amplify products in our material. Even more so, only four of the primers developed by Zini and Komjanc (2008) amplified a limited, as opposed to a smeared, product in the representative of *Pilosella* that was included in the present study, indicating that primers developed for the regional population of one species of that genus may not be widely applicable to other geographically distant species of *Pilosella*. If it is a general pattern that microsatellite markers developed for *Hieracium* s.s. are applicable to a wider range of taxa than markers designed for *Pilosella*, then this pattern is consistent with the observation that *Pilosella* appears to be more genetically diverse than *Hieracium* s.s. (Judith Fehrer and Dolores Lledo pers. comm.).

Since the markers described here are the first microsatellite markers developed for *Hieracium*, this study opens new possibilities to study the genetic structure, diversity and evolution of this intriguing and extremely diverse group of apomictic polyploids. Six out of the ten polymorphic (Table 1) microsatellites also amplified fragments in one representative of the genus *Pilosella*, and therefore several of these markers may turn out to be also applicable for molecular work in that relatively intensively studied genus. Furthermore, microsatellites yielding products from both north European representatives of *Hieracium* s.s. and from members of *Pilosella* are likely to be useful for studies throughout *Hieracium* s.l., and should even be considered for closely related genera.

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