

Development and transferability of microsatellite markers in *Habenaria nuda* and *H. repens* (Orchidaceae)

Aline Amália do Vale¹ · Bruna Ladeira Lau¹ · Bárbara Simões Santos Leal² · João Aguiar Nogueira Batista¹ · Eduardo Leite Borba³ · Karina Proite¹

Received: 29 April 2015 / Accepted: 3 September 2015 / Published online: 14 September 2015
© Botanical Society of Sao Paulo 2015

Abstract We report here the first set of microsatellite markers developed for species of *Habenaria*. A set of microsatellite markers for *Habenaria nuda* Lindl. and *H. repens* Nutt. were isolated, characterized, and tested for cross-amplification between these congeneric species. These species constitute two of the many existing complexes of the genus *Habenaria*. A total of nine markers were isolated from both species and characterized in two populations of each, with some of the markers being successfully cross-amplified. The numbers of alleles per locus ranged from four to 12 in *H. nuda*, and the mean numbers of alleles per locus were 6.0 and 6.14 in each of the populations analyzed. The observed (H_o) and expected (H_e) heterozygosities within the two populations varied from 0.294 to 0.850 and from 0.517 to 0.851, respectively. The numbers of alleles per locus ranged from four to seven in *H. repens*, and the mean numbers of alleles per locus were 2.8 and 4.0 in each of the populations analyzed. The H_o ranged from 0.000 to 0.563, and the H_e ranged from 0.000 to 0.666. The polymorphism levels found indicate these markers as powerful tools for future population genetic analyses in these and related species and could be used to address ecological, evolutionary, and taxonomic questions.

Keywords Genetic diversity · *Habenaria* · Microsatellite markers · SSR · Transferability

Introduction

Habenaria Willd. (Orchidaceae, Orchidoideae, Orchidinae) is one of the largest terrestrial orchid genera and has a pantropical distribution. The genus is well distributed in the tropics and subtropics of both the New and Old World, with only a few species extending into temperate zones (Pridgeon et al. 2001). It is especially rich in the Neotropics, Africa, and Asia, with an estimated total of 876 species (Govaerts et al. 2014). Brazil stands out as the most species rich country in the Neotropics, with 165–170 taxa (Batista et al. 2011a).

The existence of species complexes is commonplace in plants, and very frequent in orchids, where they are often the result of natural hybridization events (e.g., Ståhlberg 2009; Pinheiro et al. 2010). Such events have been reported many times in the Orchidaceae, even between species of distinct genera. Crossings between different species followed by introgression can lead to the development of individuals with intermediate morphologies, making the work of taxonomists considerably more difficult, principally because of the blurring of morphological discontinuities between taxa. Notwithstanding, other factors can also lead to species complex formation. Phenotypic plasticity in response to environment variables is one of the more important factors (Schlichting and Levin 1986), especially in terms of vegetative features, with convergence being seen as a possible response to selective pressure.

The taxonomy of *Habenaria* is particularly difficult because of the large number of species and several species complexes in the genus. In the Neotropics, the *H. nuda*

✉ Aline Amália do Vale
alineavale@gmail.com

¹ Departamento de Botânica, Universidade Federal de Minas Gerais, Caixa Postal 486, Belo Horizonte, MG 31270-901, Brazil

² Departamento de Ecologia, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus Rio Claro, Rio Claro, SP 13506-900, Brazil

³ Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Campus São Bernardo do Campo, São Bernardo do Campo, SP 09606-070, Brazil

Lindl. and *H. repens* Nutt. species complexes are among the most diverse, due to the broad geographical distribution and number of related taxa. In a synopsis of New World *Habenaria*, Batista et al. (2011a, b) listed seven synonyms and 14 other taxa as morphologically similar to *H. repens*. In turn, the *H. nuda* complex is composed of about 20 species and morphotypes (J.A.N. Batista personal comm./observation). So far, morphology alone has been unable to solve relationships in these and other complexes in the genus. A molecular phylogenetic analysis of New World *Habenaria* using nuclear (ITS) and plastid (*matK*) DNA sequences (Batista et al. 2013) confirmed some of the relationships previously based on morphology; however, it was not able to solve taxonomic relationships within these species complexes. This analysis also revealed that *H. repens* and *H. nuda* belong to different subclades of the Neotropical clade and are not directly related; however, sequence divergence among the Neotropical taxa of *Habenaria* was low.

We report here the isolation and characterization of new microsatellite markers from *H. nuda* and *H. repens*, and discuss cross-transferability between them. These markers can be very helpful in evolutionary studies, including those seeking to delimit particular species complexes, and for studies of genetic conservation. We are unaware of any earlier publications regarding microsatellite data or transferability in species of *Habenaria*.

Materials and methods

Plant material and DNA extraction

To construct a microsatellite-enriched library, total DNA was extracted from *Habenaria nuda* and *H. repens*, using a version of the 2× CTAB protocol of Doyle and Doyle (1987). In order to characterize the microsatellite markers isolated in both species, we also extracted DNA from silica gel-dried leaves of 40 individuals of *H. nuda* from two populations, named as HNUI (Moeda-MG, Brazil: 20°16'24.2"S, 43°57'29.6"W) and HNUII (Catas Altas-MG, Brazil: 20°72'9.3"S, 43°28'20.8"W), and 36 individuals of *H. repens* from two populations, named as HREI (Anguera-BA, Brazil: 12°11'44"S, 39°9'55"W) and HREII (Diamantina-MG, Brazil: 18°17'49"S, 43°48'13"W). The distance between sampled populations of *H. nuda* and between sampled populations of *H. repens* are 53.4 and 830 km, respectively.

Development of microsatellite markers

Following the protocol of Provan and Wilson (2007), 5'-anchored microsatellite primers (CCGG(AG)₈,

GCGC(AG)₈, GGCC (AC)₈, CCGG(AC)₈, and GCGC(AC)₈) were used in individual ISSR-polymerase chain reactions (PCR) that gave clear multiband products in the initial screening of *H. nuda* and *H. repens*. PCR amplifications were performed in a MJ96 Thermocycler. The general PCR system consisted of a total volume of 20 µL containing 5–15 ng genomic DNA, 0.4 µM of each primer, 1× PCR Buffer (Green Buffer Promega), 200 µM of each dNTP (Promega, USA), 2.5 mM MgCl₂, and 1.0 U *Taq* polymerase (GoTaq, Promega, USA), and water (completing 20 µL). Cycling conditions consisted of an initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

The 20 µL PCR products from each amplification were resolved on 1.5 % agarose gel in TAE buffer. Fragments between 400 and 1200 bp were extracted and purified using the MinElute Gel Extraction Kit (Qiagen, Germany), and 5 ng of the eluted DNA was ligated into the plasmid vector pGEM-T Easy Vector System (Promega, USA) according to manufacturer's instructions. Following transformation into competent cells (*Escherichia coli* XL1-Blue), 160 positive (white) clones of *H. nuda* and 200 positive clones of *H. repens* were sequenced to confirm the presence of microsatellite sequences. The identifications of microsatellite sequences and primer design were carried out using WebSat software on a web-based interface (<http://wsmartins.net/websat/>) (Martins et al. 2009), and then tested for reproducible amplification.

PCR amplification, genotyping, and data analysis

PCR amplifications of each microsatellite region were performed in a MJ96 Thermocycler, with the following cycling conditions: initial denaturation at 94 °C for 5 min, 25 cycles of denaturation at 94 °C for 30 s, annealing for 45 s at specific temperatures (according to each region; see Table 1), extension at 72 °C for 45 s, 13 cycles of denaturation at 94 °C for 30 s, annealing for 45 s at 50 °C, extension at 72 °C for 45 s, and a final extension at 72 °C for 60 min.

A M13 tail (TTTTCCCAGTCACGAC) was added to the 5' end of the forward specific primer of each marker (Schuelke 2000). PCR fragments labeled with fluorescent dyes were generated in a reaction performed with three primers: a sequence-specific forward primer with a M13 tail at its 5' end, a sequence-specific reverse primer, and the universal fluorescent-labeled M13 primer. To make multiplex reactions possible, two fluorophores (HEX and FAM) were used for different regions. The general PCR system consisted of 5–15 ng genomic DNA, 1× IB Buffer (Phonutria Biotech, Belo Horizonte, Brazil), 0.04 µM of

Table 1 Characterization of microsatellite loci in *Habenaria nuda* (NUD) and *H. repens* (REP)

Locus	Primer sequences (5'-3')	Repeat motif	Allele size Range (bp)	<i>H. nuda</i> (N = 40)		<i>H. repens</i> (N = 35)		GenBank accession
				Ta (°C)	Na	Ta (°C)	Na	
<i>H. nuda</i>								
NUD1	F: CCTCTTCCCGATCGACTTCA ^a R: AATGCAATGAAGCCGAGCAG	(TCT)8	173–209	60	10	–	–	KR085978
NUD2	F: TGTGCGAGTGAGAGAGAGAAAG ^a R: ACAATCAACAGAGTGGTTGCAC	(TG)7	326–371	60	12	55	7	KR085979
NUD3	F: CAATTTCTTGCTCCGCACTC ^a R: ACACATACGCAGATACATACGTATACACAC	(TG)8	247–295	60	12	–	–	KR085980
NUD4	F: ATCTACCATGATCTGCACCACA ^a R: GTTATAGGCGATGTTCTCGACC	(ATG)4 N5 (GGA)4	321–366	60	7	54.5	3	KR085981
NUD5	F: TGTCCGTGAATGATTCCTCA ^a R: CATCCGAGCAGTCCAATTTT	(CT)7	178–214	60	12	–	–	KR085982
NUD6	F: ACAGAAAATGTGAAGGAAGGGA ^a R: GGGAGGGAGATAGAGAGGAGAG	(TCT)4	330–348	53	5	–	–	KR085983
<i>H. repens</i>								
REP1	F: CATATCCATGTCTTCTGCC ^a R: CTCCCGTTTGCTTATCTTTTG	(CT)5CC(CT)5CC	281–305	–	–	60.7	4	KR085984
REP2	F: GGCATGGAAAAGGGTGGACTTAT ^a R: TGAGATCAACAAGGATGTCGAA	(TG)6	157–173	–	–	62.2	4	KR085985
REP3	F: GGGCCGCAGAAAATCTA ^a R: ATGTCTCCAAGCAGAGAAAAGC	(TCC)4T(TCC)	125–192	54	4	54.5	4	KR085986

Ta annealing temperature, N sample size, Na number of allele per locus

^a M13 tag (TTTTCCCAGTACAGAC) added to 5' end of forward primer for amplification with fluorescently labeled M13

forward-M13 tail primer, 0.16 μ M of reverse primer, 0.16 μ M fluorescent-labeled M13 primer, 200 μ M of each dNTP, 1 U *Taq DNA Polymerase* (Phonutria Biotech, Belo Horizonte, Brazil), and water to a final volume of 30 μ L. PCR products were checked on 1 % agarose gel, and those showing bands were size-separated by capillary electrophoresis in an ABI 3730XL automatic sequencer (Applied Biosystems) at Macrogen Inc., and sized in accordance to 500LIZ or 400HD size standards.

The ability of the developed microsatellite markers to cross-amplify between the two species was tested using the PCR and cycling conditions described above, except for the annealing temperatures, which were specific for each region and are detailed in Table 1.

The results of the fragment analyses were verified using a Peak Scanner (Applied Biosystems) and two matrices including all successfully amplified loci by each species were constructed. Individuals showing amplification fails for two or more loci were excluded. The alleles were named using macro FlexiBin2 (Amos et al. 2007) for Excel (Microsoft Office). Multiloci genotypes were identified using macro MsTools (Park 2001) in Excel. The presence of errors due to null alleles,

stuttering, or the dominance of minor alleles was checked using Microchecker 2.2.3 software (van Oosterhout et al. 2004). We estimated the number of alleles per locus (Na), observed heterozygosity (Ho), and expected heterozygosity (He) for each population and locus according to the Hardy–Weinberg equilibrium, using GenAIEx 6.4 (Peakall and Smouse 2006). The within-population inbreeding coefficients (F_{is}) were determined using FSTAT 2.9.3.2 (Goudet 1995), and null alleles' frequencies (NULL) were inferred according to Brookfield 1 method (Brookfield 1996) using Microchecker. The linkage disequilibrium among loci in each species was verified using FSTAT. Deviations from Hardy–Weinberg equilibrium were evaluated using exact tests, as implemented by GENEPOP on the Web (Raymond and Rousset 1995).

Results and discussion

The microsatellite isolation protocol used resulted in low frequencies of microsatellite-containing sequences. Out of 360 sequenced clones, only 28 contained 14 microsatellites

with motifs equal to or greater than eight dinucleotide repeats, five trinucleotide repeats, and one tetranucleotide repeat. Primers isolated from *H. nuda* and *H. repens* were named as NUD and REP, respectively. Nine of these *loci* were polymorphic (six *loci* isolated from *H. nuda* and three isolated from *H. repens*; Table 1) and showed consistent amplifications following optimization. The numbers of alleles per locus ranged from four to 12 (Table 1), with an average of six and four alleles per locus in *H. nuda* and *H. repens*, respectively (Table 2, 3). Three out of the nine isolated markers showed successful cross-amplification: one (REP3) in *H. nuda*, and two (NUD2 and NUD4) in *H. repens*.

Table 2 summarizes the genetic diversity of two populations (HNUI and HNUII) of *H. nuda* based on the analysis of seven polymorphic *loci* (six primers isolated from *H. nuda*, and one primer transferred from *H. repens*). The observed heterozygosity in the HNUI population ranged from 0.500 to 0.850 (mean 0.686), and the expected heterozygosity ranged from 0.573 to 0.813 (mean 0.683). The H_o of the HNUII population ranged from 0.294 to 0.750 (mean 0.571), and the expected heterozygosity ranged from 0.517 to 0.851 (mean 0.661) (Table 2). Among the seven *loci* analyzed in the HNUI population, three (NUD2, NUD5, and REP3) showed significant deviation from a Hardy–Weinberg equilibrium ($P < 0.005$), as did two *loci* (NUD3, NUD6) in the HNUII population (Table 2). Two *loci*, NUD3 and NUD6, showed evidence for null alleles' presence in HNUII population (see estimation of null alleles' frequencies in Table 2).

Table 3 summarizes the genetic diversity of two populations (HREI and HREII) of *H. repens* based on the analysis of five polymorphic *loci* (three primers isolated

from *H. repens* and two primers transferred from *H. nuda*). The observed heterozygosity in the HREI population ranged from 0.000 to 0.467 (mean 0.144), and the expected heterozygosity ranged from 0.000 to 0.633 (mean 0.166). The H_o of the HREII population ranged from 0.067 to 0.563 (mean 0.394), and the expected heterozygosity ranged from 0.064 to 0.666 (mean 0.486) (Table 2). Among the five analyzed *loci*, two (REP3 and NUD2) showed significant deviation from a Hardy–Weinberg equilibrium ($P < 0.005$) in the HREI population, as did one (NUD4) in the HREI population (Table 2). Only one locus, REP3, showed evidence for null alleles' presence in HREI population and two other *loci*, REP1 and NUD4, in HREII population (see estimation of null alleles' frequencies in Table 3).

The deficiencies of heterozygotes in some populations of *H. nuda* and *H. repens* could be explained by the occurrence of null alleles at those *loci*, as indicated by Microchecker. No linkage disequilibrium was observed between any two *loci* in either species.

This work reports the first set of microsatellite markers developed for species of *Habenaria*. Generally, studies on the transferability of SSR markers in orchids have presented different results, some show higher levels of transferability (e.g., Pellegrino et al. 2001; Bory et al. 2008; Pinheiro et al. 2009) and others not (e.g., Jantasuriyarat et al. 2012). Transferability of microsatellites is related to the genetic relatedness, which comprise differences in DNA sequence, genome size, and evolution rate among the species examined (Xie et al. 2010). In plants, especially monocots, it was observed that the transferability of polymorphic markers is likely to be successful mainly within genera (Barbará et al. 2007). The successful primer

Table 2 Results of primer screening for seven microsatellites in two populations of *Habenaria nuda*

Locus	HNUI population N^a	N_a	H_o	H_e	F_{is}	NULL	HNUII population N^b	N_a	H_o	H_e	F_{is}	NULL
NUD1	20	7	0.800	0.756	-0.032	-0.029	20	6	0.750	0.751	0.027	0.029
NUD2	20	7	0.500	0.672*	0.280	0.079	20	9	0.700	0.851	0.202	0.072
NUD3	20	9	0.850	0.813	-0.021	0.011	20	8	0.500	0.792*	0.391	0.146
NUD4	20	5	0.600	0.573	-0.022	-0.022	20	7	0.600	0.586	0.002	-0.002
NUD5	20	9	0.750	0.728*	-0.004	0.068	20	7	0.450	0.546	0.201	0.069
NUD6	20	3	0.500	0.646	0.226	0.089	17	3	0.294	0.517*	0.456	0.147
REP3	20	3	0.800	0.591*	-0.330	-0.109	20	4	0.700	0.584	-0.174	-0.061
Mean (SE)	20 (0)	6.143 (0.962)	0.686 (0.056)	0.683* (0.033)	0.021	0.012	19.571 (0.429)	6 (0.816)	0.571 (0.062)	0.661* (0.050)	0.163	0.057

N sample size, N_a number of allele per locus, H_o observed heterozygosity, H_e expected heterozygosity, F_{is} within inbreeding coefficient, NULL null alleles' frequency (Brookfield 1)

* Deviation from Hardy–Weinberg equilibrium ($P < 0.05$)

^a Serra da Moeda, Moeda-MG: 20°16'24.2"S/43°57'29.6"W

^b RPPN Santuário do Caraça, Catas Altas-MG: 20°7'29.3"S/43°28'20.8"W

Table 3 Results of primer screening for five microsatellites *loci* in two populations of *Habenaria repens*

Locus	HREI population N ^a	<i>Na</i>	<i>Ho</i>	<i>He</i>	<i>F_{is}</i>	<i>NULL</i>	HREII population N ^b	<i>Na</i>	<i>Ho</i>	<i>He</i>	<i>F_{is}</i>	<i>NULL</i>
REP1	19	2	0.053	0.051 ^{NA}	0.000	-0.001	15	5	0.467	0.642	0.240	0.273
REP2	20	1	0.000 ^{NA}	0.000 ^{NA}	-	0	16	4	0.375	0.520	0.125	0.013
REP3	20	2	0.000 ^{NA}	0.095*	1.000	0.087	16	5	0.563	0.537	-0.015	-0.045
NUD2	15	7	0.467	0.633*	0.295	0.102	15	2	0.067	0.064	-0.017	0
NUD4	20	2	0.050	0.049 ^{NA}	0.000	-0.001	16	4	0.500	0.666*	0.140	0.087
Mean (SE)	18.800 (0.970)	2.800 (1.068)	0.114 (0.089)	0.166* (0.118)	0.341	0.037	15.600 (0.245)	4 (0.548)	0.394 (0.087)	0.486 (0.109)	0.120	0.066

N sample size, *Na* number of allele per locus, *Ho* observed heterozygosity, *He* expected heterozygosity, *F_{is}* within inbreeding coefficient, *NULL* null alleles' frequency (Brookfield 1) *NA* Not available information

* Deviation from Hardy–Weinberg equilibrium ($P < 0.05$)

^a Anguera-BA 12°11'44"S/39°9'55"W

^b Diamantina-MG 18°17'4"S/43°48'13"W

transferability between the two *Habenaria* species examined can probably be explained by low sequence divergences among Neotropical species of that genus (Batista et al. 2013), indicating the potential application of this technique in other Neotropical *Habenaria* species. These markers can be highly informative, and the polymorphism levels found indicate them as powerful tools for future population genetic analyses in these and related species that can be used to address ecological, evolutionary, and taxonomic questions.

Acknowledgments The authors would like to thank ICMBio (Sistema SISBIO) and the Instituto Estadual de Florestas (IEF) for collection permits. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG). AAV received fellowship grants from CAPES and CNPq. BLL and BSSL received fellowship grants from CNPq. KP received a fellowship Grant from CAPES (PNPD #02805/09-0). JANB and ELB were supported by productivity Grants from CNPq (PQ2 and PQ1B, respectively).

References

- Amos W, Hoffman JI, Frodsham A, Zhang L, Best S, Hill AVS (2007) Automated binning of microsatellite alleles: problems and solutions. *Mol Ecol Notes* 7:10–14
- Barbará T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Mol Ecol* 16:3759–3767
- Batista JAN, Bianchetti LB, González-Tamayo RG, Figueroa XMC, Cribb PJ (2011a) A synopsis of new world *Habenaria* (Orchidaceae) I. *Harvard Papers Bot* 16:1–47
- Batista JAN, Bianchetti LB, González-Tamayo RG, Figueroa XMC, Cribb PJ (2011b) A synopsis of new world *Habenaria* (Orchidaceae) II. *Harvard Papers Bot* 16:233–273
- Batista JAN, Borges KS, De Faria MWF, Proite K, Ramalho AJ, Salazar GA, van den Berg C (2013) Molecular phylogenetics of the species-rich genus *Habenaria* (Orchidaceae) in the New World based on nuclear and plastid DNA sequences. *Mol Phylogenet Evol* 67:95–109
- Bory S, Silva DD, Risterucci AM, Grisoni M, Besse P, Duval MF (2008) Development of microsatellite markers in cultivated vanilla: polymorphism and transferability to other vanilla species. *Sci Hortic* 115:420–425
- Brookfield JFY (1996) A simple new method for estimating null allele frequency from heterozygote deficiency. *Mol Ecol* 5:453–455
- Doyle JF, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15
- Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-statistics. *J Hered* 86:485–486
- Govaerts R, Pfahl J, Campacci MA, Holland Baptista D, Tigges H, Shaw J, Cribb P, George A, Kreuz K, Wood J (2014) World Checklist of Orchidaceae. The Board of Trustees of the Royal Botanic Gardens, Kew. <http://apps.kew.org/wcsp/>. Accessed 2014 Dec 06
- Jantasuriyarat C, Ritsuay S, Pattarawat P, Huehne PS, Kate-Ngam S (2012) Development and transferability of EST-SSR and transferability of genomic SSR markers for genetic diversity assessment of *Doritis*. *Biochem Syst Ecol* 45:57–65
- Martins WS, Lucas DCS, Neves KFS, Bertoli DJ (2009) WebSat—a web software for microsatellite marker development. *Bioinformatics* 3:282–283
- Park SDE (2001) Trypanotolerance in West African cattle and the population genetic effects of selection. Ph.D. thesis, Trinity College Dublin, Dublin
- Peakall R, Smouse PE (2006) GenAEx 6: genetic analysis in excel. Population genetic software for teaching and research. *Mol Ecol Notes* 6:288–295
- Pellegrino G, Cafasso D, Widmer A, Soliva M, Musacchio A, Cozzolino S (2001) Isolation and characterization of microsatellite loci from the orchid *Serapias vomeracea* (Orchidaceae) and cross-priming to other *Serapias* species. *Mol Ecol Notes* 1:279–280
- Pinheiro F, Palma-Silva C, Barros F, Cozzolino S (2009) Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*. *Genet Mol Biol* 32:337–339
- Pinheiro F, Barros F, Palma-Silva C, Meyer D, Fay MF, Suzuki RM, Lexer C, Cozzolino S (2010) Hybridization and introgression across different ploidy levels in the Neotropical orchids

- Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae). Mol Ecol 19:3981–3994
- Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (2001) Genera Orchidacearum Vol. 2: Orchidoideae (part 1). Oxford University Press, New York
- Provan J, Wilson P (2007) Development of microsatellites for the peat moss *Sphagnum capillifolium* using ISSR cloning. Mol Ecol Notes 7:254–256
- Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Hered 86:248–249
- Schlichting CD, Levin DA (1986) Phenotypic plasticity: an evolving plant character. Biol J Linn Soc 29:37–47
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. A poor man's approach to genotyping for research and high-throughput diagnostics. Nat Biotechnol 18:233–234
- Ståhlberg D (2009) Habitat differentiation, hybridization and gene flow patterns in mixed populations of diploid and autotetraploid *Dactylorhiza maculata* s.l. (Orchidaceae). Evol Ecol 23:295–328
- van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538
- Xie WG, Zhang XQ, Cai HW, Liu W, Peng Y (2010) Genetic diversity analysis and transferability of cereal EST-SSR markers to orchardgrass (*Dactylis glomerata* L.). Biochem Syst Ecol 38:740–749