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De Novo Discovery and Multiplexed Amplification of Microsatellite Markers for Black Alder (*Alnus glutinosa*) and Related Species Using SSR-Enriched Shotgun Pyrosequencing

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Recent developments in sequencing technologies and bioinformatics analyses provide an unprecedented opportunity for cost and time effective high quality microsatellite marker discovery in nonmodel organisms for which no genomic information is available. Here, we use shotgun pyrosequencing of a microsatellite-enriched library to develop, for the first time, microsatellite markers for *Alnus glutinosa*, a keystone tree species of European riparian woodland communities. From a total of 17 855 short sequences, we identified 590 perfect microsatellites from which 392 had designed primers. A subset of 48 loci were tested for amplification, 12 of which were polymorphic in *A. glutinosa*. These 12 loci were successfully coamplified in a single multiplex polymerase chain reaction experiment and validated for population genetics applications. In addition, 10 and 8 of these microsatellites were found to be transferable to the related *A. incana* and *A. cordata* species. The developed multiplex of 12 microsatellite markers therefore provides new opportunities for experimental evolutionary and forest genetics research in *Alnus*.

Key words: *common alder, 454 GS-FLX Titanium, high-throughput genotyping, next-generation sequencing, short sequence repeats*

Black alder, *Alnus glutinosa* (L.) Gaertn, is an important keystone tree species that plays a key ecological role in European riparian woodlands (McVean 1953) and has multiple uses in riparian and floodplain woodland restoration as well as in forestry, horticulture, and timber industries (Krstinic et al. 2002). It is a monoecious, self-incompatible wind-pollinated tree bearing seed adapted to water dispersal, although seed dispersal by wind at smaller distance is possible (McVean 1956). Despite the well-recognized economic and ecological importance of *A. glutinosa* (Krstinic et al. 2002), there is a surprising lack of genomic resources currently available for the species. This lack of resources has greatly limited our ability to describe patterns of genetic diversity and reproduction for *A. glutinosa* to inform ecological restoration and genetic resource management programmes. The postglacial history of *A. glutinosa* is relatively well known thanks to King and Ferris (1998) who used a combination of chloroplast genetic markers and pollen fossil data to identify glacial refugia for the species. However, the availability of highly polymorphic codominant genetic markers, such as microsatellites, would allow further research on the reproductive ecology and contemporary phylogeography of *A. glutinosa*, which remain as yet virtually unknown. Such research could in turn be used to inform genetic resource management for the species. In particular, availability of microsatellite markers would allow delineation of provenance regions, which is essential in forest management of *A. glutinosa*, a species very sensitive to planting in unsuitable sites (Krstinic et al. 2002). The availability of microsatellite markers transferable to the related species *A. incana* and *A. cordata* would further assist breeding and selection programs for horticulture and forestry applications.

In this brief communication, we describe the development of new microsatellite markers for *A. glutinosa*, using cutting-edge shotgun pyrosequencing of a microsatellite-enriched library (Malausa et al. 2011). Following Lepais and Bacles (2011), we take advantage of the high number of microsatellites identified to use stringent criteria during the primer design

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phase to facilitate the optimization of a highly multiplexed microsatellite genotyping protocol (Guichoux et al. 2011) for time and cost-effective high-throughput genotyping of *A. glutinosa* and closely related species *A. cordata* and *A. incana*.

Materials and Methods

Microsatellite Discovery

Two leaves each from 12 *A. glutinosa* individuals were collected across 3 populations of the Tay river catchment (Scotland, UK) and silica dried for storage until DNA extraction with Isolate Plant DNA mini kit (Bioline) according to manufacturer instructions. Twenty-four microgram of genomic DNA was prepared using an equimolar mixture of DNA from the 12 individuals and sent to Genoscreen (Lille, France) for microsatellite-enriched library preparation and sequencing by 454 Genome Sequencer FLX Titanium (454, Roche Applied Science) using the method detailed in Malausa et al. (2011). In short, an adapted biotin-enrichment protocol was applied using 8 biotin-labeled oligonucleotides—(AG)10, (AC)10, (AAC)8, (AGG)8, (ACG)8, (AAG)8, (ACAT)6, and (ATCT)6—and the resulting library was sequenced on a 454 sequencing platform.

Sequence reads were received in the form of a FASTA file and analyzed using the QDD pipeline v1.3 (Meglécz et al. 2010) to identify reads containing simple sequence repeats and subsequently to design primers for polymerase chain reaction (PCR) amplification of microsatellite loci. We used default parameters except for the primer design step, where we used stringent parameters to select candidate marker loci that are more likely to amplify in homogeneous PCR conditions, to facilitate subsequent multiplex PCR optimization. Expected PCR product lengths were set between 90 and 400 bp with an interval of 50 bp, optimal primer pair annealing temperature of 63 °C (range 60–66 °C), 50% GC content (range 40–60%), and optimal primer length of 24 bp (range 21–30 bp).

We applied the primer screening strategy detailed in Lepais and Bacles (2011) to target loci that can be easily amplified in a multiplexed PCR. We restricted our selection to AG and AC motif types and selected candidate loci across 6 expected PCR product group size (group 1: 100–150 bp; 2: 150–200 bp; 3: 200–250 bp; 4: 250–300 bp; 5: 300–350 bp; and 6: 350–400 bp) to optimize chances of homogeneously distributed PCR product size in the selection available for PCR multiplex design. We first selected an initial set of 24 loci, 4 in each group size, with a preference for loci with the highest number of repeats to optimize polymorphic content. The second selection of an additional set of 24 loci followed the same criteria except that more loci were selected within the size groups that gave a low amplification success in the first screen. In addition, primer sequences selected for the second screen were checked against primer sequences of successful microsatellites in the first screen to limit interaction between primers during PCR due to sequence complementarities. This potential issue was checked using Multiplex Manager (Holleley and Geerts 2009) where selected loci for the second screen which primers showed interaction with validated primers in the first screen were replaced by other candidate loci.

The amplification of each of the 48 selected microsatellites was tested on a panel of 18 *A. glutinosa* trees in 7 simplex PCR reactions. Six PCR reactions used a DNA template of one individual only to check for profile interpretability and repeatability. The seventh PCR reaction consisted of a mixture of DNA from 12 individuals used as an amplification template to further characterize polymorphism, including the number of alleles and allele size range observed at each locus to help with subsequent PCR multiplex design. Simplex PCR reactions were performed using cost-effective M13 fluorescent labeling following Schuelke (2000), in a final volume of 12.5 µl using 1× MyTaq Mix (Bioline), 1 ng of template DNA, 4 pmol of the reverse primer, 1 pmol of forward primer added of the M13 sequence in 5′, and 4 pmol of M13 primer labeled with one of the fluorescent dyes 6-FAM (Eurofins MWG Operon), PET, VIC, or NED (Applied Biosystem). PCR cycles were performed in a Veriti thermocycler (Applied Biosystem) with a denaturing step of 5 min at 94 °C followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s; and then 8 cycles of 94 °C for 30 s, 53 °C for 45 s, and 72 °C for 45 s and a final elongation step of 10 min at 72 °C (Schuelke 2000). PCR products were visualized on 3.5% agarose gel (Bioline) prepared in Tris Borate Ethylenediaminetetraacetic acid (TBE) 1× and 4 µl of amplicon mixed with 1 µl of 5× Blue Loader (Bioline) with electrophoretic migrations performed at 300v for 15 min. Ethidium bromide staining was used to visualize bands under ultraviolet light. Finally, 4 µl of PCR product labeled with 4 different dyes were pooled and send for fragment analysis on an ABI 3730xl capillary sequencer at DNA Sequencing and Services (Dundee, UK) using a 1:20 dilution before run and GeneScan 500 LIZ internal size standard (ABI). Electropherogram profiles were subsequently read using STRand (Toonen and Hughes 2001).

PCR Multiplex Design and Loci Validation

We used Multiplex Manager (Holleley and Geerts 2009) and primer sequence, and allele range of loci validated in simplex PCR to determine the best set of loci to combine in a multiplexed amplification protocol. Twelve validated loci were tested for amplification in one multiplex using a panel of 36 *A. glutinosa* trees sampled in one population located 5 km south of Pitlochry (Scotland; lat N 56° 39′ 34″, long W 3° 40′ 32″), and 11 individuals randomly selected for blind repeat genotyping. Transferability of the microsatellites and of the multiplexed amplification protocol were further tested on 2 *A. incana* (L.) Moench and 2 *A. cordata* (Loisel.) Duby trees planted in parks (respectively, University of Stirling campus and Holyrood Park, City of Edinburgh). The multiplexed PCR reaction was carried out in a final volume of 5 µl using 1× Type-it Microsatellite PCR Master Mix (Qiagen), using a range of concentrations (Table 1) for each of the 12 fluorescent forward primer labeled with one of 6-FAM (Eurofins MWG Operon), VIC, PET, or NED (Applied Biosystems) dyes and reverse

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Table 1 Characteristics of 12 microsatellite primers developed in *Alnus glutinosa* and optimized to coamplify in a single multiplexed PCR

Loci	Motif	GenBank accession	Primer sequences	Dye	Primers (nM)
Ag01	$(tc)_{15}$	JF313802	F: CAGTCTATCTGCTACAAGCGTGGT R: GACGTTTTCAACGACCAAAAACAC	PET	150
Ag05	$(ga)_{12}$	JF313806	F: AAGCAAAATCCCAAGGTATCCAGT R: GGGGTTCCAACCAATTTATTCTTC	FAM	150
Ag09	$(tc)_{12}$	JF313810	F: GATGGTAATGTGACGTGAGCAAAA R: CCTATTCTCATCGTTTAAAGCCCC	NED	150
Ag10	$(tc)_{11}$	JF313811	F: AACTTGTCTTATTGTGCACTTGCG R: ACATTTACGGCTAAACAGCATTCC	FAM	150
Ag13	$(tg)_{12}$	JF313814	F: CAAGCGAAATAGATTCGTGGTCTT R: CTTCCATTTGGAGCCTTAAAACAC	VIC	150
Ag14	$(ga)_{11}$	JF313815	F: CAACCAACAAGGAGACAGAAACAA R: TAAAATCTAACACCCCAAACGAGG	FAM	500
Ag20	$(tc)_{9}$	JF313821	F: GGTTCCAAGTGGTAAGGGGAGTTA R: GAGTGTGAGAATGTGGTTCACGAG	NED	150
Ag23	$(tg)_{9}$	JF313824	F: GGTTGGGCGAAAGTTTTATTTACAC R: CCAGAAACGAACTAAGGCTAAGAAGA	PET	250
Ag25	$(ag)_{11}$	JF313826	F: GGATAAGAAGATAAAGGTGCATGGC R: CTGTATATCCCCACCACACCTGA	NED	100
Ag27	$(ag)_{9}$	JF313828	F: CATTTGGTGTATGTGTTGCCAGTT R: AATCAACAACTGCCAGGTAGAGGA	VIC	50
Ag30	$(tc)_{8}$	JF313831	F: GGAACTCTGGAAACAGAAACAACG R: AGCAAGGTAAAACTTCAGTAGCCG	FAM	100
Ag35	$(ag)_{10}$	JF313836	F: CACGTTCAGCTTCATTGTGACTTC R: TAATAGGGTTTGGGCCAACTTACC	VIC	150

For each primer pair, repeated motif type, GenBank accession number, forward and the reverse primer sequence, fluorescent dye added to the 5′ end of the forward primer and final primer concentration in the PCR mixture are given.

unlabeled primer and 5 ng of template DNA. PCR cycle were performed in a Veriti thermocycler (Applied Biosystems) consisting of a denaturing step of 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 58 °C for 180 s, and 72 °C for 30 s and a final elongation step of 30 min at 60 °C. Amplicons were checked on 3% agarose 1× TBE electrophoresis, and 3 µl of PCR product were sent for fragment analysis on an ABI3730xl capillary sequencer to DNA Sequencing and Services (Dundee,

Table 2 Characteristics of the 12 microsatellites selected for high-throughput genotyping of *Alnus glutinosa* populations

													Genotyping error rate	
Loci	N_{ind}	N_a	PIC	PI	PE_{SP}	PE_P	PE_C	He	Ho	Fis	$null_{IIM}$	N_{rep} (%)	E1 (CI 95%)	E2 (CI 95%)
Ag01	36	6	0.72	0.079	0.364	0.544	0.731	0.77	0.69	0.10	0.06	10 (28)	0.00 (0.00–0.10)	0.00 (0.00–0.07)
Ag05	35	9	0.84	0.042	0.477	0.650	0.827	0.84	0.77	0.08	0.05	10 (29)	0.00 (0.00–0.10)	0.00 (0.00–0.07)
Ag09	36	7	0.79	0.067	0.392	0.572	0.758	0.79	0.81	−0.02	0.03	10 (28)	0.00 (0.00–0.11)	0.00 (0.00–0.07)
Ag10	35	7	0.56	0.198	0.177	0.353	0.548	0.56	0.63	−0.12	0.03	10 (29)	0.00 (0.00–0.13)	0.00 (0.00–0.07)
Ag13	36	11	0.86	0.029	0.536	0.701	0.868	0.86	0.83	0.03	0.04	9 (25)	0.00 (0.00–0.12)	0.00 (0.00–0.08)
Ag14	34	9	0.82	0.054	0.437	0.614	0.794	**0.82**	**0.59**	**0.28****	**0.13****	9 (26)	0.00 (0.00–0.30)	0.00 (0.00–0.08)
Ag20	36	3	0.27	0.539	0.035	0.129	0.222	0.27	0.25	0.07	0.08	9 (28)	0.00 (0.00–0.77)	0.00 (0.00–0.08)
Ag23	36	3	0.65	0.196	0.203	0.344	0.493	0.65	0.58	0.10	0.06	9 (25)	0.00 (0.00–0.16)	0.00 (0.00–0.08)
Ag25	36	8	0.58	0.217	0.178	0.330	0.502	0.58	0.50	0.14	0.06	10 (28)	0.00 (0.00–0.11)	0.00 (0.00–0.07)
Ag27	36	5	0.54	0.239	0.150	0.303	0.466	0.54	0.47	0.13	0.08	10 (28)	0.00 (0.00–0.16)	0.00 (0.00–0.07)
Ag30	36	6	0.72	0.112	0.303	0.477	0.661	0.72	0.75	−0.04	0.03	10 (28)	0.00 (0.00–0.11)	0.00 (0.00–0.07)
Ag35	36	11	0.87	0.027	0.547	0.710	0.876	0.87	0.78	0.10	0.06	10 (28)	0.00 (0.00–0.10)	0.00 (0.00–0.07)

Number of genotyped individuals (N_{ind}); number of alleles (N_a); He, Ho, and Fis tested by 10 000 permutations of alleles within population (after Bonferroni correction for multiple tests;$^{**}P < 0.01$) were computed in Genetix (Belkhir et al. 2004). Polymorphic information content (PIC) was estimated in Cervus (Kalinowski et al. 2007). Exclusion probabilities and identity probabilities were obtained using FaMoz (Gerber et al. 2003); PI: unbiased probability of identity (combined PI over the 12Plex: <0.0000005 obtained with 6 loci or more); PE_{SP}: Exclusion probability single parent (combined PE_{SP} over the 12Plex: 0.9926); PE_P: Exclusion probability for paternity (mother known; combined PE_P over the 12Plex: 0.9998); and PE_C: Exclusion probability for a parent pair (combined PE_C over the 12Plex: >0.9999995 obtained with 10 loci or more).Null allele frequency ($null_{IIM}$) estimated with INEst (Chybicki and Burczyk 2009) using the individual inbreeding model (estimates significantly different from zero: $^{**}P < 0.01$). Per allele genotyping error rates computed in Pedant (Johnson and Haydon 2007) using a number of repeated genotyping (N_{rep} and percentage over the total number of individual genotyped for each loci N_{ind}) with E1 referring to allelic dropout rate and E2 other error rate and the 95% confidence interval (CI) computed based on He. Significant values are highlighted in bold

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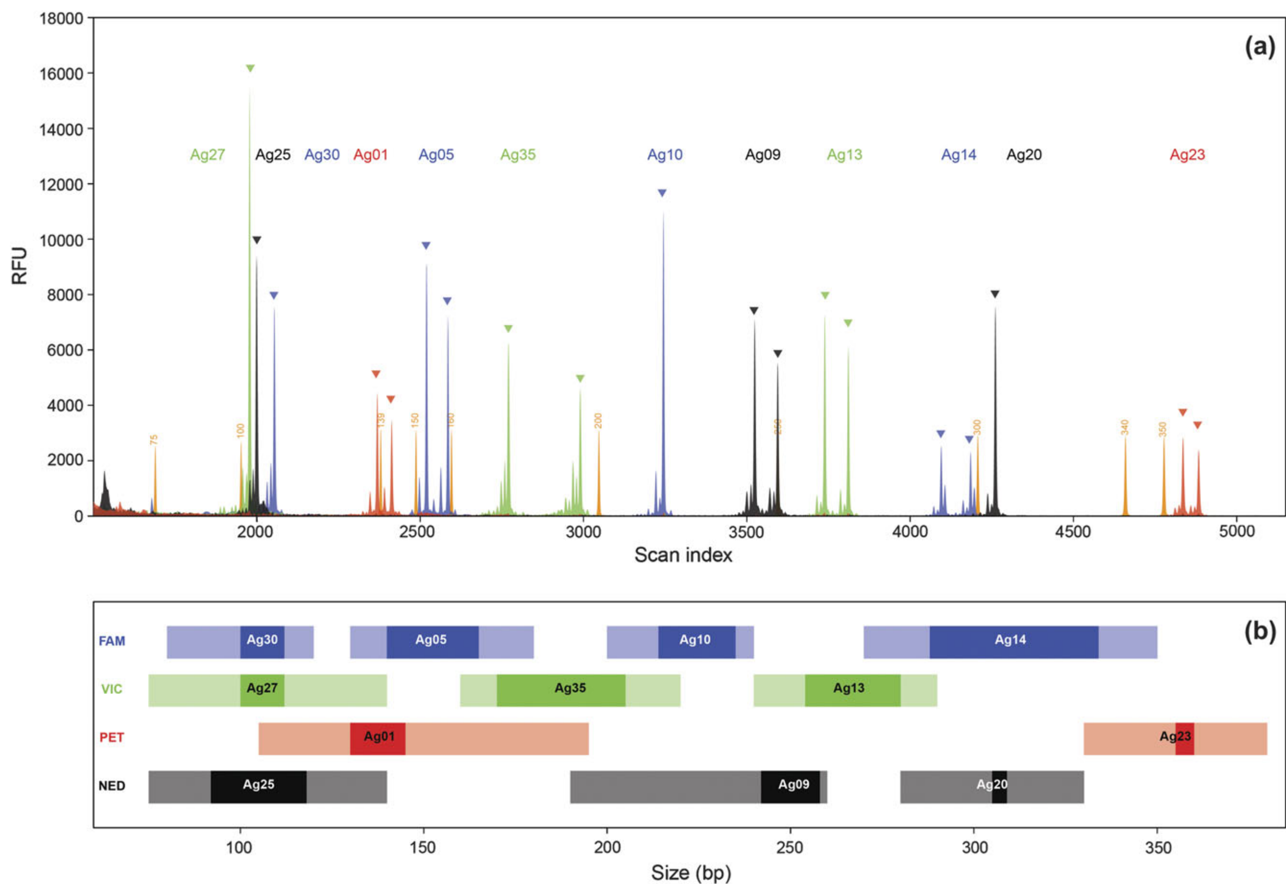

Figure 1. Example of a typical electropherogram profile obtained with the multiplexed PCR protocol for one individual (**a**) and diagram showing allele size range and fluorescent dyes of each of the 12 multiplexed loci (**b**). In (**a**), triangles indicate alleles at each locus; fragment sizes (base pair) of the 500 LIZ internal size standards are indicated by numbers above each corresponding peak. In (**b**), dark rectangles represent the observed allele range in 36 *Alnus glutinosa* individuals; light rectangles represent an arbitrary potential allele size range used during the multiplex design to avoid allele overlap of loci labeled with the same fluorescent dye.

UK) with a 1:50 dilution before run and using GeneScan 500 LIZ internal size standard (Applied Biosystems). Fluorescence profiles were analyzed using STRand and exported to MsatAllele package (Alberto 2009) in R version 2.12.0 (R Development Core Team 2010) to attribute peaks to suitable allele bin range. The suitability of selected loci for population genetic analyses was assessed by computing several diversity and information content parameters and estimating 95% confidence interval for genotyping error rate using repeated blind genotyping of 28% of the test panel (Table 2).

Results and Discussion

A total of 17 855 reads with an average length of 251 bp were obtained from 454 pyrosequencing of a microsatellite-enriched library of *A. glutinosa* (NCBI Sequence Read Archive accession number SRR099221) of which 5588 (31.3%) unique and consensus sequences containing microsatellite motif were identified (Supplementary Figure 1). Among these sequences, 590 qualified for primer design, which was successful for 392 candidate microsatellites (2.2% of the starting number of reads; Supplementary Table 1 for detailed information). As expected, the most commonly found motifs were those used for library enrichment (Supplementary Figure 2, Lepais and Bacles 2011), in particular, dinucleotide types AG and AC (193 and 75 microsatellites, respectively), followed by trinucleotides AAG, AAC, and AGG (50, 23, and 13 microsatellites). However, although AT was not used as a motif for enrichment, 25 AT microsatellites were identified. Various tri- and tetranucleotides account for the remaining 13 microsatellites (Supplementary Figure 2). Focusing on AG and AC motifs, the average number of repeated motif was 8.5 for AG and 8.3 for AC with a maximum of 18 and 45, respectively (Supplementary Figure 3).

Among the 48 candidate loci tested (GenBank Accession JF313802–JF313849), 21 failed to amplify, 6 gave inconsistent electrophoretic patterns, 9 showed no or low polymorphism levels (Supplementary Table 1) while 12 showed interpretable, clear, repeatable and polymorphic patterns and were validated

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(Table 1). All 12 loci were successfully combined in a single multiplexed PCR (Figure 1b) that after only small adjustments of primer concentrations (Table 1) yielded very clear and balanced electrophoretic profiles (Figure 1a). Ten and 8 of 12 microsatellites also amplified successfully in *A. incana* and *A. cordata*, respectively. Specifically, Ag23 and Ag25 failed to amplify in *A. incana*, whereas Ag09, Ag14, Ag23, and Ag25 failed to amplify in *A. cordata*. Most of the successfully amplified loci were found to be polymorphic in both species, even though only 2 individuals of each species were genotyped (Supplementary Table 2).

A test genotyping of 36 black alder from one Scottish population showed that these loci have a low genotyping error rate, all of them giving exactly repeatable genotypes with an observed error rate of 0.00 given wide 95% confidence intervals due to the relatively limited number of repeated genotypes (Table 2). Only Ag14 showed a significant heterozygosity deficit and significant null allele frequencies estimated at 13% (Table 2). There is therefore strong evidence for the presence of null alleles at locus Ag14 in the studied population, although the presence of null alleles is not necessarily detrimental to the estimation of population genetics parameters (Slavov et al. 2005). Overall, the 12 loci show relatively high genetic diversity (from 0.27 to 0.87, average: 0.69, Table 2), polymorphic information content (from 0.27 to 0.87, average: 0.68), and high combined exclusion probabilities for identity, paternity, and parentage analyses (Table 2).

This set of microsatellite loci, combined in a single PCR multiplex thus provides a reliable, time, and cost-effective tool to acquire genetic data most needed for a wide range of evolutionary and forestry applications in *A. glutinosa*. Additionally, the microsatellite multiplex can be used for applications in related *Alnus* species as shown by successful amplification in *A. incana* and *A. cordata* species. Indeed, the allele sizes observed in the related species are similar to those in *A. glutinosa* indicating that the same multiplexed amplification protocol can be applied for all species without fear of overlapping alleles between microsatellites labeled with the same fluorescent dye (Supplementary Table 2, Figure 1). Furthermore, the number of transferable loci is consistent with the phylogenetic relationship of these 3 species. Indeed, *A. glutinosa* and *A. incana* are more related species pairs than *A. glutinosa* and *A. cordata* are Chen and Li (2004), which translate into 10 and 8 transferable loci, respectively. Most importantly, as loci not transferable to *A. incana* are not amplified in *A. cordata* either, a minimum set of 8 loci can be used to study the 3 species jointly. This may provide a useful tool to resolve the taxonomic difficulty associated with these species (Ren et al. 2010) and more generally open new opportunities to study hybridization phenomena between European *Alnus* species (McVean 1953; King and Ferris 2000; Banaev and Bažant 2007). It should be emphasized that numerous candidate loci (provided in Supplementary Table 1) remain to be tested and could provide additional markers for *A. glutinosa* and related species.

The present study complements a growing body of literature (e.g., Santana et al. 2009; Dubut et al. 2010; Lepais and Bacles 2011; Malausa et al. 2011) and provides additional evidence of the usefulness of second-generation sequencing of microsatellite-enriched library to develop new microsatellite marker in nonmodel organisms in a matter of weeks and at relatively low expense compared with traditional methods. The method is particularly powerful in that it provides a high number of candidate loci that enables high level of multiplexing (Guichoux et al. 2011) provided that multiplex design is planned simultaneously with microsatellite development (Lepais and Bacles 2011). Such highly multiplexed microsatellite genotyping protocols greatly reduce the laboratory and expense burden and put microsatellite genetic data collection within reach of any project.

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