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Identification of 37 microsatellite loci for *Anthophora plumipes* (Hymenoptera: Apidae) using next generation sequencing and their utility in related species

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Abstract. Novel microsatellite markers for the solitary bee, *Anthophora plumipes*, were identified and characterised using 454 GS-FLX Titanium pyrosequencing technology. Thirty seven loci were tested using fluorescently labelled primers on a sample of 20 females from Prague. The number of alleles ranged from 1 to 10 (with a mean of 4 alleles per locus), resulting in an observed heterozygosity ranging from 0.05 to 0.9 and an expected heterozygosity from 0.097 to 0.887. None of the loci showed a significant deviation from the Hardy-Weinberg equilibrium and only two loci showed the significant presence of null alleles. No linkage between loci was detected. We further provide information on a single multiplex PCR consisting of 11 of the most polymorphic loci. This multiplex approach provides an effective analytical tool for analysing genetic structure and carrying out parental analyses on *Anthophora* populations. Most of the 37 loci tested also showed robust amplification in five other *Anthophora* species (*A. aestivalis*, *A. crinipes, A. plagiata*, *A. pubescens* and *A. quadrimaculata*). The result of this study demonstrates that next generation sequencing technology is a valuable method for isolating quality microsatellites in non-model species of solitary bees.

INTRODUCTION

Bees (Hymenoptera: Apoidea) are the most important group of pollinating insects (Klein et al., 2007) and in a time of global concern over the decline in the abundance of honeybees (*Apis melifera* Linnaeus, 1758) other bee species become increasingly important to humans as alternative and more resistant pollinators (Winfree, 2008). Anthophorid bees are an important group of pollinators of flowers and fruit trees (Stone, 1994; Westrich, 1989). Although there is growing evidence of both the recent, rapid population decline of many bee species and the related upcoming global pollinator crisis (Biesmeier et al., 2006; Brown & Paxton, 2009; Potts et al., 2010; Brown, 2011), the underlying genetic processes and their consequences in wild bee populations remain largely unknown. One of the reasons for this is a general lack of genetic markers for non-model organisms. Although the microsatellite loci of stingless bees are well studied (e.g., Francini et al., 2010; Francisco et al., 2011; Pereira et al., 2011) and there is a lot of literature on microsatellites in bumblebees (Estoup et al., 1995, 1996; Funk et al., 2006, Stolle et al., 2009), microsatellites have only been developed for 10 species of solitary bees (Paxton et al., 1996, 2003, 2009; Mohra et al., 2000; Kukuk et al., 2002; Azuma et al., 2005; Zayed, 2006; Souza et al., 2007; Soro & Paxton, 2009; Lopez-Uribe, 2011) and as a consequence there are few studies on the population structure and other biological aspects of these bees.

One of the reasons for the lack of genetic markers for non-model organisms is the cost and investment in time required for their development. Although the recent 454 pyrosequencing methods represent a good alternative to the traditional way of microsatellite development, reading thousands of sequences at once and discovering a huge number of microsatellite loci in one run, the use of pyrosequencing for microsatellite isolation remains rare (Malausa et al., 2011).

Malausa et al. (2011) presented a new method for high-throughput microsatellite isolation by combining DNA enrichment procedures with 454 GS-FLX technology using Titanium chemistry. We decided to take advantage of the commercially available technology for microsatellite isolation to increase the number of markers for species of solitary bees by identifying a set of new microsatellite markers for the solitary bee, *Anthophora plumipes* (Pallas, 1772). This method also provides potential genetic tools for other *Anthophora* species, including *A. plagiata* (Illiger, 1806), *A. crinipes* Smith (1854), *A. pubescens* (Fabricius, 1781), *A. quadrimaculata* (Panzer, 1798) and *A. aestivalis* (Panzer, 1801). The first two species listed are critically endangered in the Czech Republic (Farkač et al., 2005). Genus *Anthophora*, with approximately 350 species, is the largest genus of the tribe Anthophorini and lives in cosmopolitan locations worldwide, except Australia (Dubitzky, 2007). *Anthophora plumipes* is an early spring species common throughout Europe including the Czech Republic (Westrich, 1989).

Newly developed microsatellites will be further used to study the population genetics of *A. plumipes* in Europe and for a related study on the origin of introduced populations of *A. plumipes* in North America (Ascher & Pickering, 2011).

MATERIAL AND METHODS

Genomic DNA used for the microsatellite isolation was extracted from the head and thorax of one *Anthophora* female from Strahov (Prague, Czech Republic) using standard phenol-

chloroform extraction because high quality and concentrated DNA was needed. We sent 20 µl of extracted DNA (1000 ng/ml) to the Genoscreen Company (Lille, France) where all of the procedures including the DNA enrichment step (based on hybridisation with biotin-labelled oligonucleotides covering 8 different microsatellite motifs), sequence analysis and automated primer design were carried out. All of the methods used during this procedure are described in detail in Malausa et al. (2011).

From the 32,210 sequences obtained, 509 microsatellite loci with sufficient adjacent regions for primer design were acquired. Automated primer design provided us with 3,999 different primer pairs for these 509 microsatellite loci (all available on our webpage www.aculeataresearch.com). From this list of microsatellite loci and suggested primer designs, we chose 79 loci and 84 primer pairs for further amplification and polymorphism tests (for five loci two different primer pairs were chosen). The total length of the microsatellite repeat and the number of repetitions were the most important criteria for our loci-primer selection. We added the sequence GTTT to the 5´end of reverse primers with A, C or G on their 5´ends, a GTT sequence to primers with T followed by A, C or G at their 5´ends and added GT to primers ending with TT followed by A, C or G (the fluorescently labelled primers were designed in the same way). Brownstein et al. (1996) show that such a modification results in a very consistent level of PCR product adenylation and therefore fewer genotyping errors.

Initially, a sample consisting of 3 females from Strahov in Prague (Czech Republic, 50.08°N, 14.39°E) and one male from La Hoya in Murcia (Spain; 37.70°N, 01.60°W) was used to test the amplification and polymorphism of all 79 loci examined. The latter sample was chosen because male haploidy enables easier detection of possible amplification and genotyping errors and the large geographic distance of its origin from Prague greatly increased the probability of detecting polymorphisms in this 4-animal sample. The PCR reaction was performed using an Eppendorf thermal cycler and a Qiagen Type-it Microsatellite PCR Kit following the manufacturers' protocol. PCRs were carried out in 11-µl volumes consisting of 5 µl of Type-it Multiplex PCR Master Mix, 5 µl of RNAse-Free Water, 1 µl of DNA isolated from a female thorax, and a final concentration of 4.5 µM for each primer. The initial denaturation was performed at 95°C for 15 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 90 s, Tm = 63–65°C), extension (72°C for 30 s), and termination by a final extension (60°C for 30 min). All PCR products were then electrophoresed on 4% agarose gel to check for the amplification of each locus and for the presence of length polymorphism.

We subsequently ordered fluorescently labelled primers (6FAM™, VIC®, NED™ or PET®) from Applied Biosystems (further ABI) for 37 loci that amplified properly and showed any sign of polymorphism on an agarose gel. All 37 loci were tested for polymorphic content using a sample of 20 females from the Prague population. To obtain optimal results for fragment analysis, we determined that the DNA concentration in the PCR reaction should be relatively low (5–20 ng/µl) and that the optimal final primer concentration should also be low. For a PCR with fluorescently labelled primers, we therefore used 1 µl of DNA isolated from a single metatarsus and adjusted the final concentration of both reverse and forward primers to 0.18 µM, which is similar to the final concentration of each primer in a multiplex reaction, see below. The other reactants and PCR conditions remained the same, as described above. Following PCR, 1 µl of the PCR product was added to 9 µl of 95% formamide and 0.5 µl of LIZ 500 (ABI) size standard and this mixture was subsequently denatured at 90°C for 3 min. The final PCR product sizes were determined by fragment analysis on an ABI 3130 Genetic Analyzer.

Allele sizes were determined by GeneMarker 1.9 software (SoftGenetics). Program Cervus 3.0 (Marshall et al., 1998; Kalinowski et al., 2007) was used to estimate allele frequencies, observed heterozygosity (Ho) and expected heterozygosity (He). Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) was used to investigate the existence of null alleles within studied loci. Genepop (Raymond & Rousset, 1995; Rousset, 2008) was used to determine departures from Hardy-Weinberg equilibrium (HWE) for each locus, to test for linkage disequilibrium and to estimate inbreeding coefficients (FIS; according to Weir & Cockerham, 1984).

Several combinations of all polymorphic loci were tested in a single multiplex PCR on a test panel of 10 Prague individuals with already known genotypes. All loci successfully amplified in a final multiplex PCR containing 11 of the most polymorphic loci and gave repeatable genotypes identical to a single primer PCR (Table 2). All of the primers included in this multiplex were premixed together in TE buffer and the final concentration of each primer in a PCR reaction varied from 0.18 to 0.55 µM (Table 2). The other reagents in the PCR were as mentioned above. This new multiplex assay was subsequently tested on all 20 Prague females and on two distinct populations consisting of 14 females from Opava (northern Moravia, Czech Republic, 49.94°N, 17.90°E) and 13 females from Müllheim (Baden-Württemberg, Germany, 47.82°N, 7.62°E). Using the newly discovered loci, we estimated the quality of subsequent parentage and population genetic analysis by computing combined non-exclusion probabilities of all the loci used in the final multiplex using Cervus 3.0 (Marshall et al., 1998; Kalinowski et al., 2007), and also checked for the presence of null alleles in both non-Prague populations using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004).

To determine the utility of the newly described markers for genotyping other *Anthophora* species, we tested all the 37 loci on five other *Anthophora* species (2–4 individuals of each species from our personal collection) including; *A. aestivalis* (1♀, 1♂), *A. crinipes* (2♂), *A. plagiata* (2♀, 2♂), *A. pubescens* (2♂) and *A. quadrimaculata* (1♀, 1♂), using the PCR protocol and analyses described above. These taxons were selected for testing because they represent a mixture of related common (*A. aestivalis*, *A. pubescens* and *A. quadrimaculata*) and critically endangered species (*A. crinipes* and *A. plagiata*).

RESULTS AND DISCUSSION

Out of the total 79 loci tested, 64 were successfully amplified by PCR and 37 of them were further characterised using fluorescently labelled primers and fragment analysis as described above. Of these 37 loci, 36 were polymorphic and 27 contained 3–10 different alleles, with an average of 4 alleles per locus. Their characteristics are described in Table 1. Observed and expected heterozygosities were 0.05–0.9 and 0.097–0.887, respectively (Table 1) and, after Bonferroni correction, none of the loci significantly departed from HWE. Genepop (Raymond & Rousset, 1995; Rousset, 2008) detected no significant linkage disequilibrium between loci in the population studied after Bonferroni correction, and FIS ranged between –0.282 and 0.404 (Table 1). Micro-Checker (Van Oosterhout et al., 2004) did not reveal any signs of large allele dropout or stuttering at any locus and detected the presence of null alleles at only two loci: anth45 and anth71a. Nevertheless, locus anth45 was used

TABLE 1. Characteristics of 37 microsatellite loci for *Anthophora plumipes*. Motif(rep) – microsatellite motif and the number of motif repetitions, Exp. length – expected length of PCR product, Obs. length (min–max) – minima and maxima of observed lengths of PCR products in 20 females, k – number of alleles, N – number of female individuals sampled, Ngen – female individuals successfully genotyped, Ho – observed heterozygosity, He – expected heterozygosity. Heterozygosities were computed in Cervus (Marshall et al., 1998; Kalinowski et al., 2007). HW – significance of departure from HWE after applying Bonferroni correction (NS – not significant, ND – not computed), Fis – inbreeding coefficients based on the method of Weir & Cockerham (1984). The last two columns were computed in Genepop (Raymond & Rousset, 1995; Rousset, 2008).

Locus	Forward Primer (5'→3')	Reverse Primer (5'→3')	Motif(rep)	Exp. length	Obs. length (min–max)	k	N	Ngen	Ho	He	HW	Fis
anth1	AGTTCCACAAAGCATACCGC	(GTTT) CGTTCCCCACGATTAGAGAA	ttc(17)	237	225–243	3	20	20	0.65	0.627	NS	–0.038
anth8	TTAAGAACTGGACGGCGACT	(GTTT) CCTGCACTTCTGTCGTTCTG	aacgg(6)	90	85–120	7	20	20	0.55	0.645	NS	0.150
anth9	CTAACATTGCGCTCCGTGTA	(GTTT) CGTTTCGAAGAATTTCGAGG	ttc(9)	139	145–148	2	20	20	0.45	0.45	NS	0.000
anth10	GAGATGACGCGTCGAGAAAT	(GTTT) GAGGCGAGCTGCATTTTTAC	tct(9)	116	117–120	2	20	16	0.25	0.501	NS	–0.154
anth13	CCAGGTTCGTCTCAATCTCC	(GTTT) ATCGTTCATCGAGCTTGCTT	aac(9)	225	224–227	2	20	20	0.35	0.45	NS	0.227
anth14	TTTCTTTATCTTCACCACCGC	(GTTT) ACGGGTTGCAAAGTCATAGG	ctt(9)	132	132–135	2	20	20	0.4	0.492	NS	0.192
anth20	TAATTCAGCCCGGAATTTTG	(GTTT) CGTCTTTGAGAAAAGATCAGCC	gtc(8)	99	96–114	4	20	20	0.45	0.433	NS	–0.040
anth23	GCTCGCTTCCCAAATGATAA	(GTTT) ACGACGATCTCCGATTTCAC	ac(15)	148	150–154	3	20	20	0.4	0.445	NS	0.103
anth24	GATTTCGCGACTAGACCAGC	(GTTT) GACCTTCGCTACCTTCCGTA	tc(12)	112	112–122	3	20	20	0.6	0.588	NS	–0.020
anth26	CGCTTTTAACGAATTCCGAC	(GTTT) ACGTATCTCCGGGAGTCGAT	ag(13)	136	138–182	9	20	14	0.6	0.813	NS	–0.091
anth30	CAGGCAGGACAGAAACATCA	(GTTT) GTGGTCCGTCGAGTGGTATG	ca(8)	108	105–113	3	20	20	0.55	0.432	NS	–0.282
anth33	TCTGCTCTGTGAAAGCGACA	(GTTT) GGTATCGTTAAGTTCTTCCTTTTCC	ct(10)	190	191–201	5	20	20	0.8	0.696	NS	–0.154
anth35	TTGGAATCCATGTTGCACAG	(GTTT) ACATGCGTGGATGACACAGT	ttg(10)	181	176–185	2	20	20	0.1	0.097	NS	–0.027
anth37	ATCCCCACCTATCCACTTCC	(GTTT) GGGTAGTCCACTAAGCCTGC	ttc(11)	124	120–130	3	20	20	0.4	0.445	NS	0.103
anth38	TAAGTTCCTTCGATAATGAGCGT	(GTTT) AGGATGCAACTTCAGCTTTC	gtt(9)	140	144–147	2	20	20	0.25	0.296	NS	0.159
anth39	CACGACAGTACGCTGTGCTT	(GTTT) CCGTCGGATCGATAGTTTTG	ct(12)	184	179–183	3	20	20	0.35	0.422	NS	0.174
anth3a	CGATTATCGTGTTCCCCTTT	(GTTT) GCATCCGACGATAACAACAA	ttg(19)	206	204–210	2	20	20	0.2	0.185	NS	–0.086
anth40a	CAAGCCCATGTTTTCCTCTC	(GTTT) ACGAAGCGATACCACGTCTC	ca(13)	218	219–219	1	20	20	0	0	ND	ND
anth43	GAACGCAACGAATCGAAGAC	(GTT) TGGTGCATCGATAGCCTGTA	tct(19)	190	187–193	3	20	20	0.6	0.629	NS	0.048
anth44	GCGACGATTCCTACCTACCA	(GTTT) CCCCATTCTTCATTAACCGA	acct(19)	188	178–192	3	20	20	0.15	0.145	NS	–0.036
anth45	GGTTTGTACCCACGAGGAAA	(GTTT) ATTTATCCTCATTCGCTGCC	ag(14)	125	103–125	7	20	20	0.45	0.722	NS	0.383
anth47	GAGCATTTCTACATCGCCAA	(GTTT) CAATCGAGAAAGTCTGGCGT	tc(14)	148	140–172	6	20	20	0.55	0.494	NS	–0.118
anth48	CGCACCTTCGACACTACTCA	(GTT) TCGATAACGAAATAGCCACG	ac(13)	105	104–108	2	20	16	0.05	0.368	ND	ND
anth49	CCGAGTAGCGAAAGCAAGTC	(GTT) TGCGGCATTTATCAGACAAC	ac(15)	232	229–235	3	20	20	0.3	0.268	NS	–0.123
anth51	GCGTAACACGGAGAAAGGAA	(GTTT) CGCTTATTAAATGGCGCTTG	ag(13)	106	101–123	7	20	20	0.65	0.719	NS	0.099
anth52	GAAAGATTCCTCGCCACGTA	(GTTT) GATGGATTTGATTAATTGTGACGTA	ga(14)	244	241–251	5	20	20	0.75	0.722	NS	–0.04
anth54a	AGCTACCGTCCTGCTGACTC	(GTTT) CGTCCTTTCGTCGGTAAATG	ctt(22)	204	199–211	3	20	20	0.5	0.512	NS	0.023
anth55	CCTAACCGGCAATATAAACG	(GTTT) GGTATACACCTACATGTATCCATCC	ac(14)	249	249–251	2	20	19	0.3	0.559	NS	0.390
anth57	GAACCTGTACGAAGGCAAGG	(GTTT) GCATTCCAAGTCGTCGTTTT	ga(13)	96	91–99	5	20	20	0.9	0.746	NS	–0.213
anth61	GAGCGCCTAACGAATGAGG	(GTTT) AGTCAGAATACAGCCAGAATTGC	ca(16)	140	144–174	10	20	20	0.85	0.792	NS	–0.075
anth64	GATCATAAATCAACGGGTACGA	(GTT) TCGCTTAGTTACTCAACAAAGACAAC	ga(15)	147	143–165	10	20	20	0.85	0.887	NS	0.043
anth71a	TTCCACCTATTTACCTATATCGTATGC	(GTTT) CCACAGCAGCTCACCAATTT	cata(15)	140	133–162	6	20	20	0.4	0.664	NS	0.404
anth72	ATTGGACGCTTTAATGTCGG	(GTTT) AGAACTAACGAGCCAGCCAG	ttc(11)	221	223–232	4	20	20	0.5	0.541	NS	0.078
anth73	GGTTATCGAAACGTCGAGGA	(GTTT) GCACAGCCTGTAATTACGCA	gag(8)	219	218–224	3	20	20	0.3	0.476	NS	0.375
anth74	CATCTTGGGTTCGTCCAGTT	(GTTT) GTCGCCGTTACATCCGAG	tcg(14)	96	86–102	3	20	20	0.2	0.268	NS	0.259
anth75	CACCGATTTTAAACCGCTAA	(GTT) TCCGGTATCTGAACGTCAAC	ctt(10)	90	80–92	4	20	20	0.65	0.74	NS	0.124
anth76	CGAAACTAATTCTCCGCGAT	(GTT) TCGAGACAACGACCTTACCA	ctt(10)	136	138–152	4	20	20	0.7	0.55	NS	–0.282

TABLE 2. Multiplex composition and its functionality are demonstrated for three distinct populations; two from the Czech Republic (Prague and Opava) and one from Germany (Müllheim). N – individuals sampled, Mean Ngen – mean number of individuals successfully genotyped for each loci across all multiplexed loci, μM – final molar concentration of each primer in one multiplex PCR reaction, Mean Ho – mean observed heterozygosity across all loci, Mean He – mean expected heterozygosity across all loci, Mean PIC – mean polymorphic content across all loci, Combined NE-1P – combined non-exclusion probability of first parent for all 11 loci, Combined NE-2P – combined non-exclusion probability of second parent for all 11 loci, Combined NE-PP – combined non-exclusion probability of parent pair for all 11 loci, NE-I – combined non-exclusion probability of identity for all 11 loci, NE-SI – combined non-exclusion probability of sib identity for all 11 loci. All of the characteristics were computed in Cervus (Marshall et al., 1998; Kalinowski et al., 2007).

			Prague	Opava	Müllheim
N			20	14	13
Mean Ngen			20	13.9	13
Locus	Dye	μM	Number of alleles		
anth1	pet	0.18	3	3	2
anth8	vic	0.18	7	6	6
anth33	vic	0.36	5	6	3
anth45	ned	0.18	7	5	7
anth47	vic	0.55	6	8	3
anth51	pet	0.18	7	6	8
anth52	6-fam	0.36	5	7	6
anth61	pet	0.36	10	10	8
anth64	ned	0.55	10	9	5
anth75	6-fam	0.18	4	4	5
anth76	6-fam	0.18	4	3	3
Mean number of alleles			6.18	6.09	5.09
Mean Ho			0.6773	0.7144	0.5531
Mean He			0.6903	0.6915	0.5794
Mean PIC			0.6267	0.6275	0.5228
Combined NE-1P			0.0219	0.0165	0.0444
Combined NE-2P			0.0011	0.0007	0.0033
Combined NE-PP			0	0	0.0001
Combined NE-I			0	0	0
Combined NE-SI			0.0001	0.0001	0.0007

in the final multiplex analysis (Table 2) because it was highly polymorphic and there was no evidence of null alleles at this locus in the two non-Prague populations (see below).

The final, most optimal, multiplex PCR consisted of 11 primers with an average of 6.18–5.09 alleles per locus and the mean polymorphic information content (PIC) ranged from 0.52–0.63, depending on the population (Table 2). Program Micro-Checker (Van Oosterhout et al., 2004) detected the presence of null alleles in the anth45 locus in Prague populations and anth52 in Müllheim. No significant departure from HWE was detected in any of the loci assayed in this multiplex PCR for any of the populations studied. Combined non-exclusion prob-

TABLE 3. PCR results of all the 37 developed microsatellite loci for five species of the genus *Anthophora*. The numbers indicate the number of alleles detected; "–" – an unsuccessful amplification and "w" – a weaker signal in the fragment analysis. Locus 43 gave ambiguous results in most species. The number of analysed individuals: *A. aestivalis* (1♀, 1♂), *A. crinipes* (2♂), *A. plagiata* (2♀, 2♂), *A. pubescens* (2♂) and *A. quadrimaculata* (1♀, 1♂).

	Locus																		
Anth	1	8	9	10	13	14	20	23	24	26	30	33	35	37	38	39	43	44	45
A. aestivalis	1	2	–	–	–	–	1	1	2	1	1	2	1	2	1	1	1(2)	1	1
A. crinipes	1	1	2	–	2	1	1w	1	2	–	1	2w	2	2	2	2	1	2	1
A. plagiata	–	–	–	1w	–	1	–	–	2	–	1	–	1	1	–	–	1(2)	1	1
A. pubescens	–	2	1w	–	2	–	1	–	1	–	2	–	2	–	–	1	1(2)	–	1
A. quadrimaculata	1	1	3	–	1	1	2	2	1	1	1	2w	2	1	2	2	2(4)	2	2

	Locus																	
Anth	47	48	49	51	52	55	57	61	64	72	73	74	75	76	3a	40a	54a	71a
A. aestivalis	–	–	1	2w	–	–	–	2w	1	1	1	2	3	–	1	1	2	2
A. crinipes	1	1	2	1w	1	–	1	–	2	2	2	1	1	1	1	1	–	1
A. plagiata	3	–	–	–	–	–	–	–	1	2	1	1	5	–	–	2	–	1
A. pubescens	–	–	–	–	–	–	–	–	–	1	1	1	1	–	1	1	–	–
A. quadrimaculata	2	1	1	1w	2	–	1	–	3	2	2	2	1	2	2	2	1	1

abilities were generally low (0.02–0.04 for first parent, <0.001 for parent pair), which shows the sample was of high quality, which justified the use of this multiplex assay for subsequent population-genetic and parentage analyses.

Nearly all of the loci were successfully amplified in at least one other *Anthophora* species (Table 3), 9 loci amplified in all the species tested, although locus anth43 gave ambiguous results in four of the five species and needs to be optimised before use. Most loci were polymorphic in at least one species, although our sample consisted of only 2–4 animals. This study shows that it is highly probable that these microsatellite loci are widely applicable as markers across the genus *Anthophora*.

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