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Original article (Orijinal araştırma)

Success of DNA extraction and PCR amplification from dry pinned sand bees (*Andrena* spp. Fabricius, 1775) using newly-designed primers

İğnelenerek kurutulmuş kum arılarından (*Andrena* spp. Fabricius, 1775) DNA eldesi ve yeni tasarlanmış primerler kullanıldığında PCR amplifikasyonu başarısı

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Abstract

The suitability of dry pinned museum specimens for DNA extraction of sand bees (*Andrena* spp. Fabricius, 1775) (Hymenoptera: Andrenidae) and the effectiveness of existing and new primers used in DNA analysis of specimens for future studies were evaluated. A total 256 specimens were analyzed, including 222 dry pinned bee specimens representing 37 subgenera and 101 species and 34 ethanol-preserved specimens belonging to 21 species. Several different protocols were tested for DNA extraction, and DNA was extracted from almost all of the specimens. The samples preserved in ethanol had the highest quality DNA. Of 31 primer sets tested for amplification of the DNA, 14 of them were newly designed or redesigned. The amplified sequence length ranged from 130 to 1571 bp. DNA from 32 specimens belonging to 25 species was successfully amplified at three to four loci. This study demonstrates the importance of storage conditions for specimens possibly destined for later DNA extraction, and for selecting suitable primers when dealing with older bee specimens. Some primers can be diagnostically informative provided appropriate gene regions are used.

Keywords: *Andrena*, DNA, molecular, museum specimens, sand bees

Öz

İğnelenerek kurutulmuş kum arısı müze örneklerinin (*Andrena* spp. Fabricius, 1775) (Hymenoptera: Andrenidae) DNA ekstraksiyonu için uygunluğu incelenmiş ve gelecekteki çalışmalarda DNA analizi amacıyla yeni primerlerin etkinliği değerlendirilmiştir. Otuz yedi altcins ve 101 türü temsil eden 222 iğnelenmiş kuru arı örneği ve etanol içerisinde saklanmış 21 türe ait 34 arı örneği olmak üzere toplamda 256 örnek analiz edilmiştir. DNA ekstraksiyonu için birkaç farklı protokol denenmiş ve örneklerin tamamından DNA izole edilmiştir. En yüksek kaliteli DNA etanol içerisinde saklanan örneklerden elde edilmiştir. DNA amplifikasyonunda test edilen 31 primerden 14 tanesi ya yeni ya da yeniden tasarlanmıştır. Primerlerin sekans uzunluğu 130 ile 1571 bp arasında değişkenlik göstermiştir. Yirmi beş türe ait 32 örneğin üç-dört DNA lokusu başarılı bir şekilde çoğaltılmıştır. Bu çalışma, gelecekte DNA elde edilme ihtimali olan arı örneklerinin saklama koşullarının ve uygun primer seçiminin önemini ortaya koymuştur. Bazı primerler uygun gen bölgelerinin kullanılması şartıyla tür teşhislerini yapmaya yarayacak bilgileri sağlayabilirler.

Anahtar sözcükler: *Andrena*, DNA, moleküler, müze örnekleri, kum arıları

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Introduction

Bees (Apoidea: Hymenoptera) have an important role as pollinators in natural ecosystems and for many valuable crops (Klein et al., 2007). Because of their significance in agriculture and nature, many phylogenetic and evolutionary studies have been conducted with particular emphasis on wild bees (Praz et al., 2008; Rehan et al., 2010; Danforth et al., 2013; Schmidt et al., 2015). Molecular methods provide useful information regarding diagnostics for bee species, bee diversity, phylogeny, ecology, behavior, patterns of bee-host plant association and eusociality (Danforth et al., 2013; Hedtke et al., 2013; Woodard et al., 2015).

Freshly collected specimens are preferable for obtaining sufficient high-quality DNA for further scientific analysis. However, the destruction of native habitats means that museum specimens provide the only available samples for rare or otherwise difficult to acquire species (Schander & Halanychi, 2003). Also, entomological museum collections are rich repositories of insect fauna and provide historical data on the genetics, distribution and diversity of bee species (Strange et al., 2009). Unfortunately, the use of museum specimens can be challenging due to various factors including DNA degradation, contamination, and uncertainty related to specimen collection and preservation (Hernandez-Triana et al., 2014).

Dry insect specimens are usually held in museum collections, constituting over a million species. They are potentially a source of DNA. DNA sequence data from such specimens can provide useful information for both phylogenetic inference and taxonomic identification (Gilbert et al., 2007). DNA extracted from museum specimens has been helpful in the context of molecular-based identification of different bee species, as well as being a useful source of information for understanding the recent shifts in population structure, particularly regarding population declines of native pollinator species (Andersen & Mills, 2012).

The sand bee (*Andrena* spp. Fabricius, 1775) genus is presumed to be the largest genus of bees with over 1500 species described (Dubitzky et al., 2010). Information obtained from DNA of sand bees specimens held in museum collections is highly valuable because cryptic variation is common in the genus, and accurate identification using morphological methods is so challenging (Schmidt et al., 2015).

In this study, i) the effectiveness of DNA extraction protocols for dry pinned specimens of *Andrena* species compared to ethanol-preserved specimens were examined; and ii) new primer sets were designed for PCR amplification of targeted loci as a tool to obtain useful amplicons from samples with potentially fragmented DNA. Different mitochondrial and nuclear gene regions of the DNA were chosen that would be useful for analyzing the phylogeny of *Andrena* species. For this research, we used specimens of 101 bee species previously identified based on morphological methods.

Material and Methods

A total of 256 specimens were obtained for use from the Wild Bee Museum of Turkey (TUYAM) in Adnan Menderes University, Aydin, Turkey (Table 2). Of these, 222 of the specimens were dry pinned museum specimens of various *Andrena* bee species which were collected between 2004 and 2011 from different regions of Turkey. Collection and morphological identification of the bees were previously conducted by the senior author and Erwin Scheuchl. The bee specimens belong to 101 species from 37 subgenera. For purposes of comparison, a further 34 bee specimens that had been preserved in ethanol (96%) and belonging to 21 different species were included. All the ethanol-preserved specimens were collected in 2014 by the senior author from different areas in Turkey. All experiments were conducted at the Southeastern Fruit and Tree Nut Research Laboratory, USDA-ARS, Byron, Georgia, USA between 2014 and 2015.

DNA extraction

Qiagen DNeasy Blood and Tissue Kits (Quiagen, Redwood City, CA, USA) were used to extract DNA from the sand bee specimens following the manufacturer's protocol with three modifications (Nishiguchi et al., 2002; Ward, 2009; Crane, 2011). Nonetheless, due to difficulties with DNA extraction from certain specimens, a 2x cetyltrimethylammonium bromide (CTAB) DNA extraction method was implemented for

six bee species. The 2x CTAB DNA extraction method was that of Danforth (2013). This CTAB method takes 3 d to complete. Briefly, the DNA extraction procedure was as follows (with buffers AE, AL, ATL, AW1, AW2 and TE from the Qiagen kit). Individual dry pinned specimens were placed in Petri dishes and separated into three sections (head, thorax and abdomen) using a pair of fine forceps. The wings and legs were removed from the thorax. The bee samples preserved in 96% ethanol were washed with TE buffer for 10 min, dried on filter paper at room temperature for 30 min, and placed in a 2 ml microcentrifuge tube in a freezer at -20°C overnight. A single body part (head, thorax or abdomen) was used for DNA extraction and the remaining body parts were retained as a voucher and for further use if required. A bashing bead to crush the sample was placed in the 2 ml microcentrifuge tube with the bee body part and the sample homogenized using a Qiagen TissueLyser at 200 Hz for 1-2 min. In the second method tested, 20 samples were homogenized with a plastic pestle until they were finely ground. Proteinase K (20 µl) and buffer AL (200 µl) were added and mixed thoroughly by vortexing; the mixture was incubated at 54-56°C for 3-4 h or overnight in a water bath. The sample was vortexed for 15 s. A further 200 µl of buffer AL was added to the sample, and mixed thoroughly by vortexing. Ethanol (96-100%) (200 µl) was added and the mixture again vortexed thoroughly. The mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The flow-through was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl buffer AW1 was added, and the tubes were centrifuged at 8000 rpm for 1 min. The flow-through was discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl buffer AW2 was added, and centrifuged for 3 min at 14,000 rpm. The flow-through was discarded. The DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, and 100 µl buffer AE was pipetted directly onto the DNeasy membrane. The tubes and column were incubated at room temperature for 1 min, and subsequently centrifuged for 1 min at 8000 rpm to elute the DNA from the column. The elution was repeated once as described in previous step.

Three steps in the protocol were changed as in previous studies. In the first variation, the samples were incubated with buffer ATL and proteinase K at 56°C overnight (Nishiguchi et al., 2002). After adding a further 200 µl AL buffer, the samples were incubated at 65°C for 15 min. Then, 200 µl ethanol was added and the mixture incubated at 4°C for 1 h. Buffer AE was added to tube at 70°C. The second variation to the protocol was as described by Ward (2009), who added 180 µl buffer AL to microcentrifuge tube including the sample. The mixture was incubated 56°C for 4 h in a water bath. The tube was shaken briefly every 45-60 min. The third variations were as follows. The bee body part was placed in a 1.5 ml microcentrifuge tube and 60 µl PBS (phosphate buffered saline), 40 µl proteinase K and 200 µl buffer AL were added and mixed thoroughly by vortexing, and incubated at 56°C for 4 h in a water bath (Crane, 2011). A Nanodrop spectrophotometer was used to quantify the DNA in each sample. Extracted DNA was stored at -20°C until it was used in the PCR.

PCR amplification of DNA with primers

An adequate amount of the PCR reagent mix (without adding the sample DNA) was prepared and stored in an ice bucket for each cohort of PCR reactions, with sufficient additional mix for two samples; one as a negative control and the other as spare reagent in case of pipetting errors. The reagent mix was vortexed thoroughly.

The following reagents were used in each 10 µl PCR mix: 5 µl PCR Master Mix (Promega PCR Master Mix, 2X), containing 50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM of nucleotides (dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2), 1 µl forward primer, 1 µl reverse primer and 2 µl deionize water.

The reagent mix (9 µl) was added to each PCR tube and 1 µl of the sample was added (10 ng DNA per reaction). Dilutions of DNA samples were made based on the results from the Nanodrop measurements. The negative control contained 1 µl of ddH_2O. The tubes were placed in the PCR machine, and the appropriate PCR program (initial denaturation for 3 min at 94°C; 36 cycles of 1 min denaturation at 94°C, 1 min annealing ranging between 40 and 60°C and 1 min elongation at 72°C, and a final extension for 5 min at 72°C) was run for the marker being amplified. The annealing temperatures for each primer set are presented in Table 1. The tubes were removed from the PCR machine and stored in a refrigerator.

Seventeen primer sets were initially screened in this study. Eleven of them amplify mitochondrial gene regions and six of them amplify nuclear gene regions. However, these primers failed to produce amplicons with many samples, particularly with dry pinned bee samples. So, new primers were designed for these sand bee samples. BLAST, Primer-BLAST and Primer3 programs were used for designing new primers (Ye et al., 2012). In addition, six primers previously described in the literature were redesigned according to sequence alignments data of *Andrena* spp. or other Andrenidae species in Genbank (NCBI-NIH, MD, USA). Subsequently, an additional 14 primer sets were tested. These were newly designed or redesigned primer sets; two amplifying mitochondrial gene regions and 12 amplifying nuclear gene regions. Thus, a total of 31 primer sets were tested in this study (Table 1), with sequence lengths ranging from 130 to 1571 bp.

Table 1. Primer sets used to amplify DNA of *Andrena* species

PRIMERS		DNA sequence (bp)	Annealing temperature (°C)	References
	MITOCHONDRIAL DNA PRIMERS			
mitochondrial cytochrome oxidase I (COI)				
mtD8	For 5'-CCACATTTATTTTGATTTTTTGG-3'	853	48	Dubitzky, 2005
mtD12	Rev 5'-TCCAATGCACTAATCTGCCATATTA-3'			
AP-L-2176	For 5'-GGTACAGGTTGAACTGTTTACCC-3'	521	40	Koulianos & Schmid-Hempel, 2000
AP-H-2650	Rev 5'-TCCGACTGTAAATAAGTGATGTGCTC-3'			
LCO1490	For 5'-GGTCAACAAATCATAAAGATATTGG-3'	710	45-50	Reemer et al., 2008
HCO2198	Rev 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'			
LepFl	For 5′-ATTCAACCAATCATAAAGATAT-3'	350	45-51	Hebert et al., 2004
LepR2	Rev 5'-CTTATATTATTTATTCGTGGGAAAGC-3'			
CO1-2166F	For 5'-GGAGGATTTGGTAATTTTTTAATTCC-3'	226	45	Francoso & Arias, 2013
CO1-2386R	Rev 5'-GAAAAAATTGTAAAATCAAC-3'			
Uni-MinibarF1	For 5'-TCCACTAATCACAARGATATTGGTAC-3'	130	46-53	Meusnier et al., 2008
Uni-MinibarR1	Rev 5'-GAAAATCATAATGAAGGCATGAGC-3'			
AndCOI-F1	For 5'-TTGCYATATGAGCAGGCATAGTCG-3'	631	51	New
COIand-R1	Rev 5'-TTGGTATARAATDGGRTCTCCWCCT-3'			
AndCOI-F2	For 5'-GAGCCGGAATAATTGGTGCC-3'	615	53	New
COIand-R2	Rev 5'-GGATCGGATCTCCACCTCCTA-3'			
mitochondrial COI-COII				
Jack	For 5'-AGATCACTTGAATGATCACAAAAT-3'	695	55	Larkin et al., 2006
Barb	Rev 5'-CCACAAATTTCTGAACATTGACCA-3'			
mitochondrial cytochrome oxidase b (Cyt b)				
cb1	For 5'-TATGTACTACCATGAGGACAAATATC-3'	429	50	Rehan et al., 2010
cb2	Rev 5'-ATTACACCTCCTAATTTATTAGGAAT-3'			

Table 1. continued

	PRIMERS	DNA sequence (bp)	Annealing temperature (C°)	References
	MITOCHONDRIAL DNA PRIMERS			
mitochondrial 12S rRNA				
12Sa	For 5' TGGGATTAGATACCCCACTAT-3'	428	50	Cameron&Williams, 2003
12SLR	Rev 5'-YYTACTATGTTACGACTTAT-3'			
mitochondrial 16S rRNA				
16S-F	For 5'-TTATTCACCTGTTTATCAAAACAT-3'	600	50	Ramirez et al., 2010
16S-R	Rev 5'-TATAGATAGAAACCAATCT-3'			
16SWb	For 5'-CACCTGTTTATCAAAAACAT-3'	500	48	Hines et al., 2006
874-16SlR	Rev 5'-TATAGATAGAAACCAATCTG-3'			
	NUCLEAR DNA PRIMERS			
28S rRNA				
Bel28S	For 5'-AGAGAGAGTTCAAGAGTACGTG-3'	690	60	Danforth et al., 2006
Mar28Srev	Rev 5'-TAGTTCACCATCTTTCGGGTCCC-3'			
And28S-F	For 5'-GAGATTCAKCGTCRACGAGGCT-3'	669	60	New
28Sand-R	Rev 5'-TGACCAGGCATAGTTCACCA-3'			
EF-1 α F1 copy				
EF-1For2	For 5'-AAGGAGGC[C/G]CAGGAGATGGG-3'	457	52	Schwarz et al., 2004
EF-1Rev2	Rev 5'-[T/C]TC[G/C]AC[T/C]TTCCATCCGTACC-3'			
EF-1 *α* F2 copy				
For1-deg	For 5'-GYATCGACAARCGTACSATYG-3'	1571	52	Danforth et al., 1999
F2-Rev1	Rev 5'-AATCAGCAGCACCTTTAGGTGG-3'			
F2-ForH	For 5′-GGRCAYAGAGATTTCATCAAGAAC-3′	720	54	Hines et al., 2006
F2-RevH2	Rev 5′- TTGCAAAGCTTCRKGATGCATTT-3′			
HaF2For1-And	For 5'-GGGYAAGGGWTCCTTCAARTACGC-3'	1080	59	redesigned (Danforth et al., 1999)
F2-rev1-And	Rev 5'-AATCRGCAGCACCYTTGGGTGG-3'			
AndEF-F1	For 5'-TTACBGGYACMTCACARGCTGACT-3'	700	60	New
EFand-R1	Rev 5'-CACGRCCGACTRGTACTGTTC-3'			
AndEF-F2	For 5'-TGAGACGTGGTTACGTAGCAG-3'	538	52	New
EFand-R2	Rev 5'-GGGAACTCTTGGAAAGCCTCA-3'			

Table 1. continued

PRIMERS		DNA sequence (bp)	Annealing temperature (°C)	References
NUCLEAR DNA PRIMERS				
Opsin				
Opsin For3 (mod)	For 5'-TTCGAYAGATACAACGTRATCGTNAARGG-3'	639	56	Michez et al., 2009
Opsin Rev (mod)	Rev 5'-ATANGGNGTCCANGCCATGAACCA-3'			
Opsin For3 (mod)-And	For 5'-TTCGACAGATACAACGTRATYGTMAARGG-3'	610	58	redesigned (Michez et al., 2009)
OPSand-R1	Rev 5'-TCGAATATGCCCGACGTGTT-3'			New
AndOPS-F2	For 5'-TTCTCTCTGGGCTGGACAAT-3'	708	51	New
OPSand-R2	Rev 5'-AACAGYGCAGCTCGATACTT-3'			
ArgK				
F	For 5’-GTTGACCAAGCYGTYTTGGA-3’	860	48	Hines et al., 2006
R	Rev 5’-CATGGAAATAATACGRAGRTG-3’			
Wingless				
wgColletFor-And	For 5'-CACGTGTCBTCGGRAATGAGRCAGGA-3'	670	59	redesigned (Almeida & Danforth, 2009)
Lep wg2a-Rev	Rev 5'-ACTICGCARCACCARTGGAATGTRCA-3'			Almeida & Danforth, 2009
AndWNG-F	For 5'-ATCGGGTACGGGTTCAAGTT-3'	653	59	New
WNGand-R	Rev 5'-GTCACCTCCTGCGTCYTGTA-3'			
CAD				
ApCADfor4-And	For 5'-TGGAARGARGTBGAATTCGAAGTGAACGC-3'	684	51	redesigned (Danforth et al., 2006)
CADand-R	Rev 5'-TTCACTACCGCAGCAATCTG-3'			New
AndCAD-F	For 5'-GCTATCCSCTGGCWTACGTAGCT-3'	720	60	New
ApCADrev4a-And	Rev 5'-GGCCAYTCCGCAGCCACHGTGTCTATYTG YTTCACC-3'			redesigned (Danforth et al., 2006)
RNA polymerase II (Pol II)				
polfor2a-And	For 5'-GGAGAACTKGTGATGGGTATACTTTG-3'	587	59	redesigned (Danforth et al., 2006)
polrev2a-And	Rev 5'-AGGTACGARTTYTCAACGAATCCTCT-3'			
AndPOL-F	For 5'-AAATGACGAAGAGGGACGTG-3'	723	50	New
POLand-R	Rev 5'-CGCAAGCGATAACCTGAGAG-3'			

Agarose gel electrophoresis

After the PCR reaction was compete, 5 μl from each sample was run on a 1.5% agarose minigel against a standard size marker (Bionexus Hi-Lo™ DNA marker, Oakland, CA, USA). The sizes of the

amplicons were compared against the molecular weight marker to confirm whether the PCR reaction had amplified the target region. PCR products were purified using the QIAquick Purification Kit (Qiagen).

Results and Discussion

Several different protocols including the Qiagen DNeasy Blood and Tissue Kit, and three modifications of its protocol (Nishiguchi et al., 2002; Ward, 2009; Crane, 2011), and 2x CTAB DNA (Danforth, 2013) were compared for extraction of DNA from sand bees. The Qiagen kit was the most effective method especially for the dry pinned specimens. DNA was extracted from almost all tested specimens. However, the fresher, more recently collected ethanol-preserved samples clearly had better quality DNA compared with the dry pinned specimens. DNA was obtained from both sexes. There was no significant difference in the quantity and quality of DNA extracted from female and male bees (Tables 2 and 3).

Table 2. Quantification and quality of DNA of *Andrena* species obtained from dry pinned specimens

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
1001A	F	*Aciandrena*	*Andrena aciculata* Morawitz, 1886	all body	Qiagen	3	45.4	1.93	1.75
1001B	M			all body	Qiagen	3	17.0	1.60	1.22
1002A	M	*Aciandrena*	*Andrena lamiana* Warncke, 1965	Head, Thx	Qiagen	3	3.8	2.26	1.19
1101A	F	*Aenandrena*	*Andrena aeneiventris* Morawitz, 1872	4 Legs	Qiagen	4	1.5	2.25	0.55
1101B	F			all body	Qiagen	3	57.1	1.90	1.67
1101C	F			all body	Qiagen	3	35.3	1.84	1.74
1102A	F	*Aenandrena*	*Andrena bisulcata* Morawitz, 1877	4 Legs	Qiagen	4	11.2	1.41	0.88
				Thorax	Nishiguchi et al., 2002	over night	3639.5	1.43	0.60
1102B	F			all body	Qiagen	3	128.4	1.95	1.87
1102C	F			all body	Qiagen	3	108.1	1.97	1.70
1103A	F	*Aenandrena*	*Andrena hystrix* Schmiedeknecht, 1883	4 Legs	Qiagen	4	6.6	1.42	0.61
				Head	Nishiguchi et al., 2002	over night	2.2	1.68	1.49
1103B	F			all body	Qiagen	3	42.8	1.57	0.78
1103C	F			all body	Qiagen	3	47.3	1.66	1.09
1201A	F	*Brachyandrena*	*Andrena colletiformis* Morawitz, 1874	3 Legs	Qiagen	4	8.7	1.42	0.79
1201B	F			all body	Qiagen	3	10.7	1.83	1.07
1201C	M			all body	Qiagen	3	11.3	1.98	1.35
1301A	F	*Campylogaster*	*Andrena lateralis* Morawitz, 1876	Thx, abd	Qiagen	3	18.8	1.56	0.63
1401A	F	*Carandrena*	*Andrena falcinella* Warncke, 1969	all body	Qiagen	3	86.7	1.89	1.72
1401B	F			all body	Qiagen	3	42.6	1.91	2.00
1402A	F	*Carandrena*	*Andrena purpureomicans* Alfken, 1935	all body	Qiagen	3	17.7	1.63	1.15
1402B	M			all body	Qiagen	3	16.0	1.74	1.28
1402C	M			Head, Thx	Qiagen	3	11.0	1.97	0.96
1501A	F	*Charitandrena*	*Andrena hattorfiana* (Fabricius, 1775)	Thx, abd	Qiagen	3	151.3	1.89	1.73
1501B	F			Thx, abd	Qiagen	3	16.3	1.69	0.99
1601A	F	*Chlorandrena*	*Andrena cinerea* Brulle, 1832	all body	Qiagen	3	48.9	1.80	0.91
1601B	F			all body	Qiagen	3	73.7	1.88	1.63
1602A	F	*Chlorandrena*	*Andrena cinereophila* Warncke, 1965	all body	Qiagen	3	15.9	1.73	0.62
1602B	F			all body	2XCTAB	2	473.9	1.37	0.91
1602C	F			Thx, abd	Qiagen	3	42.7	1.78	0.99
1602D	F			all body	Qiagen	3	60.9	1.91	2.11

Table 2. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
1603A	F	*Chlorandrena*	*Andrena clypella* Strand, 1921	all body	Qiagen	3	126.2	1.90	1.82
1603B	F			all body	2XCTAB	2	934.7	1.40	0.62
1604A	F	*Chlorandrena*	*Andrena exquisita* Warncke, 1975	all body	Qiagen	3	66.1	1.85	1.41
1604B	F			all body	2XCTAB	2	756.5	1.40	0.56
1604C	F			all body	Qiagen	3	34.9	1.63	1.04
1604D	F			all body	Qiagen	3	181.3	1.94	1.74
1605A	F	*Chlorandrena*	*Andrena humabilis* Warncke, 1965	Thx, abd	Qiagen	3	27.9	1.74	1.01
1605B	F			all body	Qiagen	3	38.4	1.77	1.17
1606A	F	*Chlorandrena*	*Andrena humilis* Imhoff, 1832	all body	Qiagen	3	74.2	1.87	1.51
1606B	F			all body	2XCTAB	2	419.8	1.06	0.93
1606D	M			Head, Thx	Qiagen	3	44.5	1.87	1.13
1607A	F	*Chlorandrena*	*Andrena orientana* Warncke, 1965	all body	Qiagen	3	234.9	1.97	2.06
1607B	F			all body	2XCTAB	2	1086.7	1.47	0.77
1608A	F	*Chlorandrena*	*Andrena panurgimorpha* Mavromoustakis, 1957	all body	Qiagen-insects	15	61.1	1.77	1.48
1608B	F			all body	2XCTAB	2	1332.1	1.38	0.64
1608C	F			all body	Qiagen	3	111.1	1.96	2.10
1608D	M			Head, Thx	Qiagen	3	39.3	1.76	1.37
1701A	F	*Chrysandrena*	*Andrena hesperia* Smith, 1853	all body	Qiagen	3	225.9	1.96	1.82
1701B	F			all body	Qiagen	3	347.2	2.01	2.23
1702A	F	*Chrysandrena*	*Andrena merula* Warncke, 1969	all body	Qiagen	3	20.0	1.67	0.78
1702B	F			all body	Qiagen	3	69.5	1.95	1.69
1702C	F			all body	Qiagen	3	41.8	1.84	1.08
1801A	F	*Cordandrena*	*Andrena cordialis* Morawitz, 1877	all body	Qiagen	3	51.4	1.67	0.90
1801B	F			all body	Qiagen	3	40.0	1.49	0.83
1801C	F			all body	Qiagen	3	151.5	1.89	1.93
1802A	F	*Cordandrena*	*Andrena cypria* Pittioni, 1950	all body	Qiagen	3	164.7	1.91	1.67
1802B	F			all body	Qiagen	3	117.9	1.95	2.11
1901A	F	*Didonia*	*Andrena nasuta* Giraud, 1863	Thx, legs	Qiagen	3	59.8	1.81	1.14
2001A	F	*Euandrena*	*Andrena bicolor* Fabricius, 1775	all body	Qiagen	3	14.6	1.55	0.75
2001B	F			Thx, abd	Qiagen	3	253.5	1.79	1.83
2002A	F	*Euandrena*	*Andrena glabriventris* Alfken, 1935	all body	Qiagen	3	148.1	1.93	1.54
2002B	F			Thx, abd	Qiagen	3	116.6	1.95	1.93
2101A	F	*Holandrena*	*Andrena labialis* (Kirby, 1802)	all body	Qiagen	3	150.1	1.73	1.14
2101B	F			Thx, abd	Qiagen	3	84.8	1.81	1.48
2101C	F			Abdomen	Qiagen	3	17.5	1.55	0.65
2101D	M			Head, Thx	Qiagen	3	36.4	1.77	0.90
2102B	F	*Holandrena*	*Andrena variabilis* Smith, 1853	all body	Qiagen	3	15.4	1.51	0.60
2102C	M			Thx, abd	Qiagen	3	132.1	1.93	1.83
2103A	F	*Holandrena*	*Andrena wilhelmi* Schuberth, 1995	all body	Qiagen	3	27.3	1.37	0.53
2103B	F			Thx, abd	Qiagen	3	116.5	1.91	1.62
2103C	F			all body	Qiagen	3	35.8	1.64	0.55
2201A	F	*Hyperandrena*	*Andrena bicolorata* (Rossi, 1790)	all body	Qiagen	3	133.2	1.76	1.20
2201B	F			Thx, legs	Qiagen	3	83.1	1.90	1.92

Table 2. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
2301A	F	*Larandrena*	*Andrena medioxima* Warncke, 1975	Thx, abd	Qiagen	3	69.9	1.95	1.73
2302A	F	*Larandrena*	*Andrena sericata* Imhoff, 1866	all body	Qiagen	3	26.0	1.63	0.77
2302B	F			all body	Qiagen	3	204.1	1.97	2.05
2302C	M			all body	Qiagen	3	23.2	1.81	1.37
2401A	F	*Lepidandrena*	*Andrena gamskrucki eburnea* Warncke, 1975	all body	Qiagen	3	193.4	2.00	1.92
2401C	M			Head, Thx	Qiagen	3	47.9	1.91	1.55
2402A	F	*Lepidandrena*	*Andrena gamskrucki impasta* Warncke, 1975	Thx, abd	Qiagen	3	541.4	2.07	2.14
2501A	F	*Leucandrena*	*Andrena mistrensis* Grünwaldt, 2005	all body	Qiagen	3	123.9	1.76	1.38
2502A	F	*Leucandrena*	*Andrena parviceps* Kriechbaumer, 1873	Thx, abd	Qiagen	3	187.3	1.81	1.89
2601A	F	*Melanapis*	*Andrena fuscosa* Erichson, 1835	Thx, legs	Qiagen	3	331.9	2.01	1.87
2701A	F	*Melandrena*	*Andrena albopunctata* (Rossi, 1792)	Thx, legs	Qiagen	3	139.7	1.9	2.02
2701B	F			all body	Qiagen	3	290.3	1.92	1.87
2702A	F	*Melandrena*	*Andrena atrotegularis* Hedicke, 1923	Thx, legs	Qiagen	3	17.2	1.83	0.63
2703A	F	*Melandrena*	*Andrena danuvia* Stöckhert, 1950	Thx, legs	Qiagen	3	61.3	1.81	1.43
2703B	F			Thx, abd	Qiagen	3	135.4	1.85	1.43
2704A	M	*Melandrena*	*Andrena fuscocalcarata* Morawitz, 1877	Head, Thx	Qiagen	3	209.5	1.98	2.01
2705A	F	*Melandrena*	*Andrena limata* Smith, 1853	all body	Qiagen	3	125.9	0.44	0.37
2705B	F			all body	Qiagen	3	70.4	1.73	1.12
2705C	F			Thx, abd	Qiagen	3	79.4	1.76	1.40
2705D	F			Thx, abd	Qiagen	3	158.6	1.80	1.17
2706A	F	*Melandrena*	*Andrena morio* Brullè, 1832	Thx, legs	Qiagen	3	204.7	1.85	1.41
2706B	F			Thx, abd	Qiagen	3	76.5	1.68	0.94
2706C	F			Thx, abd	Qiagen	3	79.8	1.43	0.59
2706D	F			Thx, abd	Qiagen	3	47.1	1.39	0.72
2707A	F	*Melandrena*	*Andrena nigroaenea candiae* Strand, 1915	Thx, abd	Qiagen	3	29.1	1.42	0.55
2707B	F			Thx, abd	Qiagen	3	30.7	1.29	0.59
2707C	F			Thx, abd	Qiagen	3	32.1	1.47	0.62
2707D	M			Head, Thx	Qiagen	3	51.7	1.57	0.79
2708A	F	*Melandrena*	*Andrena nitidemula* Scheuchl & Hazir, 2012	Thx, legs	Qiagen	3	63.7	1.92	1.44
2709A	F	*Melandrena*	*Andrena pyropygia* Kriechbaumer, 1873	Thx, abd	Qiagen	3	29.8	1.53	0.49
2801A	F	*Melittoides*	*Andrena curiosa* (Morawitz, 1877)	Thx, legs	Qiagen	3	43.2	1.53	0.54
2901A	F	*Micrandrena*	*Andrena virgata* Warncke, 1975	all body	Qiagen	3	71.9	1.91	1.63
3001A	F	*Nobandrena*	*Andrena anatolica* Alfken, 1935	all body	Qiagen	3	218.5	1.97	1.86
3001C	F			all body	Qiagen	3	122.2	1.91	1.65

Table 2. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
3002A	F	*Nobandrena*	*Andrena athenensis* Warncke, 1965	all body	Qiagen	3	96.5	1.83	1.27
3002B	F			Thx, abd	Qiagen	3	219.0	1.91	2.23
3003A	F	*Nobandrena*	*Andrena nobilis* Morawitz, 1874	all body	Qiagen	3	138.9	1.78	1.37
3003B	F			Thx, abd,	Qiagen	3	274.5	1.95	1.96
3004A	M	*Nobandrena*	*Andrena probata* Warncke, 1973	Head, Thx	Qiagen	3	106.3	1.94	1.60
3101A	F	*Notandrena*	*Andrena langadensis* Warncke, 1965	Thx, abd	Qiagen	3	95.3	1.90	1.79
3102A	M	*Notandrena*	*Andrena recurvirostra* Warncke, 1975	Head, Thx	Qiagen	3	19.9	1.72	0.88
3103A	F	*Notandrena*	*Andrena ungeri* Mavromoustakis, 1952	all body	Qiagen	3	23.8	1.78	1.57
3103B	F			all body	Qiagen	3	72.9	1.83	1.34
3201A	F	*Opandrena*	*Andrena schencki* Morawitz, 1866	all body	Qiagen	3	52.2	1.60	0.80
3201B	F			all body	Qiagen	3	61.5	1.71	1.10
3201C	F			Thx, abd	Qiagen	3	53.8	1.69	1.07
3201D	F			Thx, abd	Qiagen	3	111.4	1.83	1.41
3301A	F	*Orandrena*	*Andrena garrula* Warncke, 1966	all body	Qiagen	3	54.0	1.86	1.70
3301B	F			all body	Qiagen	3	121.3	1.83	1.64
3401A	F	*Parandrenella*	*Andrena crispa* Warncke, 1975	all body	Qiagen	3	117.2	1.84	1.45
3401B	F			all body	Qiagen	3	153.1	1.92	1.85
3402A	F	*Parandrenella*	*Andrena dentiventris* Morawitz, 1874	all body	Qiagen	3	109.5	1.92	2.05
3402B	F			all body	Qiagen	3	83.8	1.99	2.00
3403A	F	*Parandrenella*	*Andrena figurata* Morawitz, 1866	all body	Qiagen	3	60.1	1.90	1.70
3403B	F			all body	Qiagen	3	81.8	1.94	1.84
3501A	F	*Plastandrena*	*Andrena bimaculata* (Kirby, 1802)	Thx, abd	Qiagen	3	43.3	1.38	0.60
3501B	F			Thx, abd	Qiagen	3	26.3	1.78	1.07
3501C	M			all body	Qiagen	3	23.6	1.56	0.65
3502A	F	*Plastandrena*	*Andrena pilipes* Fabricius, 1781	Thx, abd	Qiagen	3	34.3	1.53	0.72
3502B	F			Thx, abd	Qiagen	3	143.9	1.90	1.85
3502C	M			Head, Thx	Qiagen	3	69.6	1.90	1.53
3502D	M			Head, Thx	Qiagen	3	35.9	1.92	1.32
3601A	F	*Poecilandrena*	*Andrena crassana* Warncke, 1965	all body	Qiagen	3	106.0	1.94	1.70
3602A	F	*Poecilandrena*	*Andrena efeana* Scheuchl & Hazir, 2012	Thx, abd	Qiagen	3	98.3	1.86	1.52
3603A	F	*Poecilandrena*	*Andrena hybrida* Warncke, 1975	all body	Qiagen	3	119.3	1.95	1.89
3604A	F	*Poecilandrena*	*Andrena labiata* Fabricius, 1781	all body	Qiagen	3	45.4	1.92	1.66
3604B	M			Head, Thx	Qiagen	3	8.7	1.42	0.67
3605A	F	*Poecilandrena*	*Andrena laticeps* Morawitz, 1877	all body	Qiagen	3	108.4	1.91	1.79
3605B	F			all body	Qiagen	3	53.6	1.89	1.55
3605C	M			Head, Thx	Qiagen	3	28.4	1.95	1.11
3606A	F	*Poecilandrena*	*Andrena semirubra* Morawitz, 1876	all body	Qiagen	3	75.1	1.89	1.33
3607A	F			all body	Qiagen	3	97.6	1.93	1.88

Table 2. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
3701A	F	*Poliandrena*	*Andrena kriechbaumeri* Schmiedeknecht, 1883	all body	Qiagen	3	140.1	1.93	1.86
3701B	F			all body	Qiagen	3	69.7	1.89	1.14
3701C	M			Head, Thx	Qiagen	3	33.9	1.90	1.19
3702A	F	*Poliandrena*	*Andrena limbata* Eversmann, 1852	all body	Qiagen	3	27.7	1.62	0.67
3702B	F			Thx, abd	Qiagen	3	27.5	1.37	0.51
3703A	F	*Poliandrena*	*Andrena polita* Smith, 1847	Thx, abd	Qiagen	3	160.3	1.98	2.15
3703C	M			all body	Qiagen	3	35.5	1.85	1.45
3801A	F	*Proxiandrena*	*Andrena alutacea* Stoeckhert, 1942	all body	Qiagen	3	118.4	1.88	1.74
3801B	F			all body	Qiagen	3	258.5	1.96	1.85
3901A	F	*Ptilandrena*	*Andrena glidia* Warncke, 1965	all body	Qiagen	3	30.4	1.61	0.84
3902A	F	*Ptilandrena*	*Andrena vetula* Lepeletier, 1841	all body	Qiagen	3	204.7	1.92	1.83
4001A	F	*Scitandrena*	*Andrena scita* Eversmann, 1852	Thx, abd	Qiagen	3	36.4	1.53	0.73
4001B	F			Thx, abd	Qiagen	3	55.8	1.80	1.33
4001C	F			Thx, abd	Qiagen	3	32.7	1.48	0.56
4001D	M			Head, Thx	Qiagen	3	69.3	1.83	1.45
4101A	F	*Simandrena*	*Andrena combinata* (Christ, 1791)	Thx, abd	Qiagen	3	80.9	1.66	1.04
4101B	F			all body	Qiagen	3	22.8	1.66	1.15
4102A	F	*Simandrena*	*Andrena dorsata* (Kirby, 1802)	all body	Qiagen	3	102.2	1.87	1.57
4102D	M			Head, Thx	Qiagen	3	41.4	1.96	1.53
4103A	F	*Simandrena*	*Andrena lepida* Schenck, 1861	all body	Qiagen	3	227.9	1.90	2.06
4103D	F			all body	Qiagen	3	142.0	1.90	1.55
4104A	F	*Simandrena*	*Andrena transitoria* Morawitz, 1871	Thx, abd	Qiagen	3	24.5	1.47	0.80
4104B	F			Thx, abd	Qiagen	3	105.8	1.79	1.23
4104C	M			Head, Thx	Qiagen	3	34.9	1.87	1.86
4201A	F	*Thysandrena*	*Andrena ranunculorum* Morawitz, 1877	Thx, abd	Qiagen	3	175.6	1.79	1.41
4301A	F	*Trachandrena*	*Andrena haemorrhoa* (Fabricius, 1781)	all body	Qiagen	3	164.0	1.91	1.84
4401A	F	*Truncandrena*	*Andrena asiatica* Friese, 1921	all body	Qiagen	3	44.0	1.49	0.77
4401B	F			Thx, abd	Qiagen	3	30.8	1.53	0.86
4401D	M			Head, Thx	Qiagen	3	173.8	2.05	2.22
4402A	F	*Truncandrena*	*Andrena caneae* Strand, 1915	Thx, abd	Qiagen	3	179.1	2.00	2.06
4402B	M			Head, Thx	Qiagen	3	60.6	2.05	1.76
4403A	F	*Truncandrena*	*Andrena combusta* Morawitz, 1876	all body	Qiagen	3	33.2	1.58	0.89
4403B	F			Thx, abd	Qiagen	3	81.8	1.88	1.38
4404A	F	*Truncandrena*	*Andrena medeninensis usura* Warncke, 1967	all body	Qiagen	3	171.9	1.92	1.72
4404B	F			all body	Qiagen	3	111.2	1.89	1.54
4405A	F	*Truncandrena*	*Andrena optata* Warncke, 1975	all body	Qiagen	3	316.8	1.86	1.84
4405D	M			Head, Thx	Qiagen	3	34.4	1.67	0.77
4406A	F	*Truncandrena*	*Andrena roseotincta* Warncke, 1975	all body	Qiagen	3	93.7	1.83	1.20
4406C	F			Thx, abd	Qiagen	3	99.1	1.95	2.13
4407A	F	*Truncandrena*	*Andrena schmiedeknechti* Magretti, 1883	all body	Qiagen	3	147.7	1.95	1.94
4407D	M			Head, Thx	Qiagen	3	106.1	1.91	1.47

Table 2. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA (ng/µl)	260/280	260/230
4408A	F	*Truncandrena*	*Andrena seitzi* Alfken, 1935	Thx, abd	Qiagen	3	88.8	1.81	1.55
4408B	F			all body	Qiagen	3	95.5	1.82	1.59
4409A	F	*Truncandrena*	*Andrena truncatilabris* Morawitz, 1877	all body	Qiagen	3	32.0	1.40	0.69
4409B	F			all body	Qiagen-insects	15	54.4	1.64	0.99
4409C	F			all body	Qiagen	3	337.8	1.99	2.08
4409D	M			Head, Thx	Qiagen	3	45.1	1.91	1.62
4409F	M			Head, Thx	Qiagen	3	15.7	1.79	0.79
4410A	F	*Truncandrena*	*Andrena ulula* Warncke, 1969	all body	Qiagen	3	47.9	1.70	1.02
4410B	F			all body	Qiagen	3	97.1	1.88	1.73
4411A	F	*Truncandrena*	*Andrena urfanella* Scheuchl & Hazir, 2012	all body	Qiagen	3	58.5	1.73	1.08
4411B	F			all body	Qiagen	3	106.1	1.81	1.51
4411C	F			Thx, abd	Qiagen	3	96.1	1.86	1.47
4501A	F	*Ulandrena*	*Andrena cantiaca* Warncke, 1975	all body	Qiagen	3	140.1	1.93	1.81
4501D	M			Head, Thx	Qiagen	3	51.7	1.89	1.45
4502A	F	*Ulandrena*	*Andrena crecca* Warncke, 1965	Thx, abd	Qiagen	3	97.3	1.46	0.71
4502B	F			Thx, abd	Qiagen	3	77.4	1.91	1.85
4502C	M			Head, Thx	Qiagen	3	44.0	1.83	1.02
4503A	F	*Ulandrena*	*Andrena elegans* Giraud, 1863	Thx, legs	Qiagen	3	100.6	1.85	1.74
4503C	M			Head,Thx	Qiagen	3	108.4	1.60	1.19
4504A	F	*Ulandrena*	*Andrena fulvitarsis* Brullè, 1832	all body	Qiagen	3	120.7	1.89	1.71
4504B	F			all body	Qiagen-insects	15	44.3	0.95	0.70
4504E	M			Head, Thx	Qiagen	3	59.7	1.71	1.02
4505A	M	*Ulandrena*	*Andrena heinrichi* Grünwaldt, 2005	Head, Thx	Qiagen	3	129.8	1.89	1.41
4506A	F	*Ulandrena*	*Andrena neocypriaca* Mavromoustakis, 1956	all body	Qiagen	3	80.4	1.90	2.07
4506B	F			all body	Qiagen	3	42.5	1.90	1.56
4507A	F	*Ulandrena*	*Andrena osychniukae* Osytshnjuk, 1977	all body	Qiagen	3	71.5	1.97	1.52
4507B	M			all body	Qiagen	3	71.5	1.60	1.43
4507C	M			Head, Thx	Qiagen	3	26.6	1.66	1.16
4601A	F	*Zonadrena*	*Andrena flavipes* Panzer, 1799	all body	Qiagen-insects	15	114.5	1.76	1.51
4601B	F			Thx, abd	Qiagen	3	17.4	1.25	0.58
4601E	F			all body	Qiagen	3	135.7	0.59	0.52
4601F	F			all body	Ward, 2009	3	6.5	1.79	1.11
				Head, Thx	Crane, 2011	3	28.0	1.79	1.43
				Abdomen	Nishiguchi et al., 2002	over night	912.8	1.42	0.61
4601G	F			Head	Qiagen	4	82.8	1.96	2.02
4601H	M			Head, Thx	Qiagen	3	27.2	1.88	0.92
4601K	M			all body	Ward, 2009	4	24.3	1.97	1.12
				Head, Thx	Crane, 2011	4	0.4	4.95	0.13
4602A	F	*Zonadrena*	*Andrena gazelle* Friese, 1922	all body	Qiagen	3	42.4	1.74	1.19
4602B	F			Thx, abd	Qiagen	3	23.1	1.44	0.60

Thx: Thorax; Abd: Abdomen

Table 3. Quantification and quality of DNA obtained from ethanol (96%) preserved specimens of *Andrena* species

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA	260/280	260/230
1501X	F	*Charitandrena*	*Andrena hattorfiana* (Fabricius, 1775)	Thorax	Qiagen	4	284.4	1.59	1.06
1501X	F			Abdomen	Qiagen	4	373.1	1.47	0.77
1501X	F			Legs	Qiagen	4	17.0	1.45	0.63
1501Y	F			Thx, legs	Qiagen	3	456.1	2.16	2.25
1501Z	F			Thx, legs	Qiagen	3	392.1	2.14	2.19
1701X	F	*Chrysandrena*	*Andrena hesperia* Smith, 1853	Thx, abd	Qiagen	3	422.4	2.14	2.28
1701Y	F			Abdomen	Qiagen	3	216.4	2.12	2.22
1701Y	F			Thorax	Qiagen	3	111.4	2.14	2.19
1702X	F	*Chrysandrena*	*Andrena merula* Warncke, 1969	Thx, abd	Qiagen	3	471.7	2.14	2.13
1801X	F	*Cordandrena*	*Andrena cordialis* Morawitz, 1877	Thx, abd	Qiagen	3	307.7	2.06	1.75
1901X	F	*Didonia*	*Andrena nasuta* Giraud, 1863	Thorax	Qiagen	3	274.4	2.16	2.25
1901Y	F			Thx, legs	Qiagen	3	399.9	2.17	2.26
2403X	F	*Lepidandrena*	*Andrena curvungula* Thomson, 1870	Thorax	Qiagen	3	481.8	2.13	2.20
2501X	F	*Leucandrena*	*Andrena mistrensis* Grünwaldt, 2005	Thx, legs	Qiagen	3	281.3	2.12	2.13
2702X	F	*Melandrena*	*Andrena atrotegularis* Hedicke, 1923	Thorax	Qiagen	3	351.4	2.08	1.92
2703X	F	*Melandrena*	*Andrena danuvia* Stöckhert, 1950	Thorax	Qiagen	3	363.5	2.09	1.59
2703Y	F			Thx, legs	Qiagen	3	437.0	2.14	2.19
2703Z	F			Thx, legs	Qiagen	3	464.5	2.11	2.16
2710X	F	*Melandrena*	*Andrena thoracica* (Fabricius, 1775)	Thx, legs	Qiagen	3	695.1	2.12	2.26
3003X	F	*Nobandrena*	*Andrena nobilis* Morawitz, 1874	Thorax	Qiagen	3	353.6	2.15	2.16
3003Y	F			Thx, legs	Qiagen	3	512.3	2.14	2.28
3201X	F	*Opandrena*	*Andrena schencki* Morawitz, 1866	Thx, legs	Qiagen	3	379.9	2.07	1.91
3502X	M	*Plastandrena*	*Andrena pilipes* Fabricius, 1781	Thx, legs	Qiagen	3	84.8	2.03	2.09
3703X	F	*Poliandrena*	*Andrena polita* Smith, 1847	Thx, abd	Qiagen	3	564.9	2.13	2.21
3703Y	F			Thx, abd	Qiagen	3	413.3	2.14	2.19
3703Z	F			Abdomen	Qiagen	3	441.8	2.13	2.22
3703Z	F			Thorax	Qiagen	3	222.4	2.14	2.07
3703Z	F			Legs	Qiagen	3	25.0	2.20	1.36

Table 3. (Continued)

Code	Sex	Subgenus	Species	Material	DNA extraction method	Water bath incubation period (h)	Nanodrop		
							DNA	260/280	260/230
4001X	F	*Scitandrena*	*Andrena scita* Eversmann, 1852	Thorax	Qiagen	3	398.5	2.13	2.22
4103X	F	*Simandrena*	*Andrena lepida* Schenck, 1861	Thx, abd	Qiagen	3	474.4	2.15	2.24
4401X	F	*Truncandrena*	*Andrena asiatica* Friese, 1921	Thorax	Qiagen	3	386.5	2.13	2.25
4401Y	F			Thx, legs	Qiagen	3	351.5	2.15	2.24
4401Z	M			Thx, legs	Qiagen	3	133.2	2.04	1.84
4407X	F	*Truncandrena*	*Andrena schmiedeknechti* Magretti, 1883	Thx, legs	Qiagen	3	350.3	2.16	2.26
4502X	F	*Ulandrena*	*Andrena crecca* Warncke, 1965	Thorax	Qiagen	3	359.3	2.16	2.27
4502Y	F			Thx, legs	Qiagen	3	268.4	2.14	2.12
4601X	F	*Zonandrena*	*Andrena flavipes* Panzer, 1799	3 Legs	Qiagen	4	10.0	1.68	0.58
4601X	F			Thorax	Qiagen	4	250.6	1.59	0.91
4601X	F			Abdomen	Qiagen	4	692.4	1.90	1.48
4601X	F			Legs	Qiagen	4	28.0	1.44	0.67
4601Y	F			Thx, abd	Qiagen	3	278.6	2.15	2.18
4602X	F	*Zonandrena*	*Andrena gazella* Friese, 1922	Thx, legs	Qiagen	3	220.9	2.14	2.02

Thx: Thorax; Abd: Abdomen

DNA of all ethanol preserved samples was amplified with most of the primers tested (with the exception of primer pairs Uni-MinibarF1/Uni-MinibarR1, 12Sa/12SLR, ArgK-F/R, AndCOI-F1/COIand-R1 and AndCOI-F2/COIand-R2). The PCR was successful for almost all ethanol samples, whereas the amplification of DNA from only a few of the dry pinned specimens was achieved. The primers were selected that worked for both ethanol preserved and dry pinned specimens. These were used for further studies. The DNA samples were amplified by five primer sets (AndWNG-F/WNGand-R, polfor2a-And/polrev2a-And, Bel28S/Mar28Srev, And28S-F/28Sand-R, AndEF-F1/EFand-R1) and for four loci (wingless wnt-1 gene, RNA polymerase II, 28S ribosomal RNA, elongation factor 1 alpha F2 copy). DNA from 32 specimens belonging to 25 species and representing three to four loci was successfully amplified. Of these, 28 were ethanol-preserved specimens. Four of them (*Andrena gamskrucki impasta* Warncke, 1975*, A. fuscosa* Erichson, 1835*, A. gamskrucki eburnea* Warncke, 1975*, A. semirubra* Morawitz, 1876) were dry pinned specimens (Figure 1).

Preservation methods can have a direct effect on the quality and quantity of the DNA extracted from insect specimens. This study demonstrates that good quality DNA can be readily extracted and amplified from samples of sand bees preserved in ethanol. However, obtaining PCR amplifiable DNA from dry pinned specimens was difficult. None of the primers tested were reliable for amplifying DNA from the dry pinned specimens. However, five primers worked for about 2% of the dry specimens and they would be potentially useful for phylogenetic analyses. There are other factors that can affect the success of DNA extraction resulting in suitable quality DNA for PCR amplification from dry pinned specimens, including bee size, time since collection, DNA degrading contaminants, kill method and marker allele size.

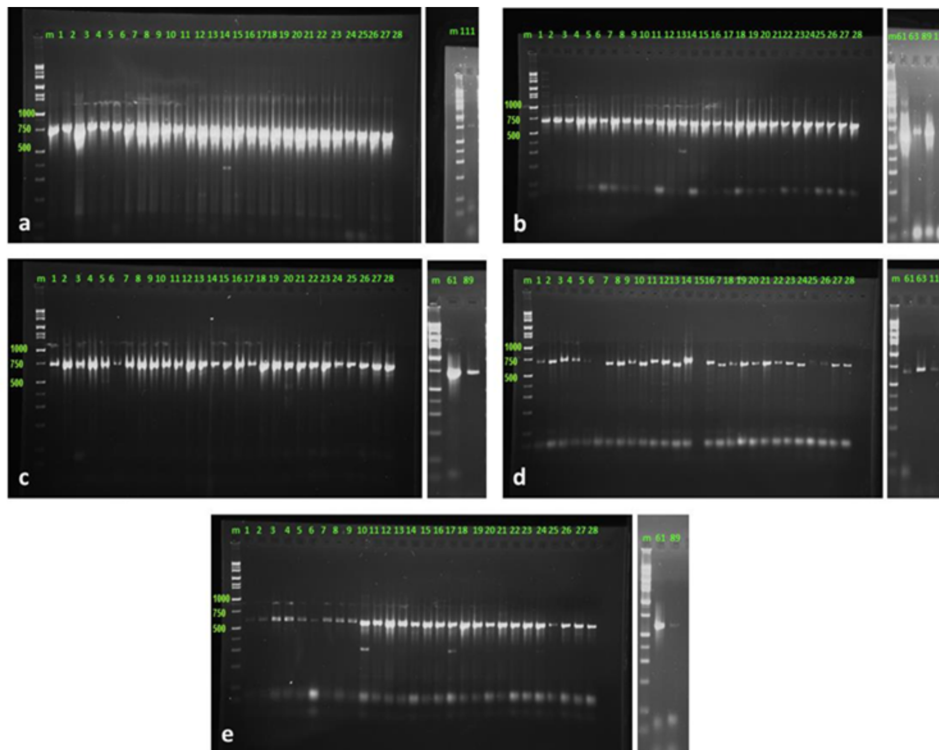

Figure 1. Agarose gel images showing products of PCR amplification of different primers for DNA sequence in *Andrena* species: a) Bel28S/Mar28Srev (~750 bp); b) And28S-F/28Sand-R (700 bp); c) AndEF-F1/EFand-R1(750 bp); d) AndWNG-F/WNGand-R (653 bp); e) polfor2a-And/polrev2a-And (587 bp) (lanes 1 to 28 contain DNA of ethanol preserved specimens; lanes 61 (2402A), 63 (2601A), 89 (2401A), 111 (3607A) contain DNA of dry pinned specimens).

Andersen & Mills (2012) reported that specimen size significantly affected the total amount of extracted DNA from a braconid parasitoid specimen. Strange et al. (2009) suggested that pinned bumble bee specimens from museum collections are suitable for population genetic studies because of their large size. In our study, the size of bee specimens ranged from small (5 mm) to medium (18 mm). The better quality DNA was obtained from larger bees, for example *A. albopunctata* (Rossi, 1792)*, A. fuscosa* Erichson, 1835*, A. fuscocalcarata* Morawitz, 1877 (Table 2). The result could be associated with both the number of cells and the thickness of exoskeleton layer. The cuticle, which is an extracellular layer that covers the complete external surface of insects and acts both as a skeleton for muscle attachment and as a protective barrier. The thickness of cuticular layer varies from a few micrometers to a few millimeters, depending upon the insect species (Andersen, 2009). Strange et al. (2009) hypothesized that sclerotization of the bumble bees in museum collections helped preserve the genetic material. It is known that insect body size is strongly correlated with cuticle thickness (Peeters et al., 2017). Considering this information, it can be suggested that the bigger bees have a thicker cuticle, which protect the cells more. This hypothesis is supported by our data for sand bees.

If no preservation steps are taken, time since death has a negative effect on the likelihood of successful DNA amplification. DNA in museum specimens generally becomes degraded and the quality and quantity remaining often precludes molecular genetic studies (Gilbert et al., 2007; Zimmermann et al., 2008; Strange et al., 2009). In our study 222 dry pinned specimens of *Andrena* bees were collected between 2004-2011. DNA was extracted from almost all the dry pinned specimens that were screened. However, successful DNA amplification was possible for only four of these specimens. Notably, in our study, two of these samples, *Andrena gamskrucki impasta* (2402A) and *A. gamskrucki eburnea* (2401A), were relatively

recent collections (2011), but other specimens collected at the same time either did not contain sufficient DNA, or the DNA was not of sufficient quality. However, DNA amplification was successful with two older museum specimens, *A. fuscosa* (2601A) and *A. semirubra* (3607A), collected in 2007. Even if DNA amplification success decreases with the time since collection, at least some successful results would have been expected for specimens collected in 2008, 2009 or 2010. It is likely that the failure to amplify DNA from the oldest specimens is likely due to postmortem degradation of DNA. However, the reason why DNA was not amplified from most of the younger specimens is unknown. It is possible that the killing methods and storage conditions of these samples may have resulted in degradation of all of the DNA. Although time is important, it is likely to be only one of the factors.

Insect specimens are usually killed with ethyl acetate, ethyl alcohol, formalin or cyanide depending on the taxon, the method of collection and the choice of collector. Specimen labels usually do not include details of the killing agent used, or the length of exposure to the killing agent. Due to these factors, DNA extraction and amplification is unlikely to be successful from all dry specimens. Also, different collection and storage conditions affect the quantity and quality of DNA (Gilbert et al., 2007). In our study, all of the specimens were killed using ethyl acetate. There are few studies that have focused on the effect of the ethyl acetate on quality of DNA. Dillon et al. (1996) suggested that specimens killed in ethyl acetate vapor had fragmented DNA that gave consistently low yields when extracted and that could not be successfully amplified. Consequently, we consider that ethyl acetate is not a suitable killing reagent if the aim is to perform molecular studies on insects. Whereas, Willows-Munro & Schoeman (2015) claimed that there was no evidence that DNA degradation depended on killing method, including use of ethyl acetate. Therefore, further research is needed to clarify this issue.

Andersen & Mills (2012) suggested that short fragments of 28S and COI genes were sufficient for species identification, and for examining within species genetic diversity. They examined DNA extraction from museum specimens of parasitic Hymenoptera (Braconidae). In their study, several primer combinations of various length were tested, but these did not amplify fragments longer than 150 bp. In our study, 31 primer sets were tested. Of these, 13 amplified mitochondrial gene regions and 18 amplified nuclear gene regions. Five primers were selected that work for both ethanol preserved bee specimens and a few of the dry pinned specimens (AndWNG-F/WNGand-R, polfor2a-And/polrev2a-And, Bel28S/Mar28Srev, And28S-F/28Sand-R, AndEF-F1/EFand-R1) for four loci (wingless wnt-1 gene, RNA polymerase II, 28S ribosomal RNA, Elongation factor 1 alpha F2 copy). Sequence lengths were 653, 587, 690, 669 and 700 bp, respectively. Shorter amplicon sequence lengths were also tested (130, 226 and 350 bp) but these failed to give useful results. Accordingly, we contend that shorter sequence length is not effective in DNA amplification for dry museum specimens.

PCR can be influenced by many conditions including the template DNA preparation and reaction conditions, and primer design (Ye et al., 2012). The primer design is an important step to ensure successful PCR. In this study, 14 primer sets were newly designed or modified based on reported primer sequence in the literature. Twelve of these resulted in successful amplification of chosen nuclear gene regions. These primers are useful for entomologists intending to use bee specimens for systematic studies.

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