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ORIGINAL ARTICLE

Genome-wide characterization of microsatellites and development of polymorphic markers shared between two weevils of *Eucryptorrhynchus* (Coleoptera: Curculionidae)

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Abstract The weevils *Eucryptorrhynchus brandti* (Harold) and *E. scrobiculatus* (Motschulsky) are two pests of the tree of heaven, *Ailanthus altissima*. They usually co-occur on their host tree. However, these two species have a niche separation with different eggs-laying locations and larvae-feeding parts on the tree. Here, we characterized the distribution of microsatellites in the genome and developed genome-wide microsatellite markers shared between these two weevils. A total of 12,524 and 11,030 potential microsatellites were identified for *E. brandti* and *E. scrobiculatus*, respectively. Microsatellites with AT, TA, AC, TG, CA repeat motifs were most common for both species. We designed primers universal for two weevils and developed 14 loci with high amplification efficacy and polymorphism. We validated these microsatellite markers in three populations of each species. In *E. brandti*, the observed and expected heterozygosity for the genotyped loci ranged from 0.17–0.24 and 0.44–0.54; in *E. scrobiculatus*, the above values ranged from 0.06–0.18 and 0.18–0.27. Based on the developed microsatellite markers, we found that populations of both weevils have a clear pattern of genetic differentiation. Our study provides valuable genetic markers for ecological, genetic, and evolutionary study of these two weevils.

Key words Microsatellite, primer, population genetics.

1 Introduction

The weevils *Eucryptorrhynchus brandti* (Harold, 1881) (EB) and *E. scrobiculatus* (Motschulsky, 1854) (ES) are two pests of the tree of heaven, *Ailanthus altissima* (Mill.) Swingle (Yang & Wen, 2021). They usually co-occur on their host tree. However, there is a micro-scale niche separation between these two closely related species (Ji *et al.*, 2017). Larvae of EB mainly feed on the trunk, while ES mainly feed on the root. Adults of EB lay eggs on the trunk, while adults of ES lay eggs on the ground near the tree. Previously, the two weevils were not primary pests of *A. altissima*. Since the past decades, they have caused increasing damages to *A. altissima*, which are important ornamental plants along the roadsides in northern China (Wu *et al.*, 2016). The boring of the larvae usually leads to the death of *A. altissima*. Due to the recent outbreaks of these two weevils, many aspects of the two species remain unclear, such as their mechanisms of niche separation, dispersal, and local adaptation.

Population genetic study can reveal the gene flow and genetic differentiation of species and may shed light on the development of sustainable pest control (Niu *et al.*, 2006). Molecular markers from the mitochondrial and nuclear genomes were usually used for population genetic studies. Mitochondrial genomes of the two species have been determined (Liu *et*

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al., 2016; Nan *et al.*, 2016). However, mitochondrial genes are maternally inherited and come from the same locus, which may not reveal the complete evolutionary history of species (Jwo & Whitlock, 2010). Nuclear markers developed from multiple loci are useful for understanding population genetic diversity and differentiation (Cao *et al.*, 2015, 2016).

A microsatellite is a tract of tandemly repeated DNA motifs that are ubiquitous in the coding and non-coding regions of the nuclear genome. Due to their hyper-variability, high repeatability, and co-dominance, microsatellites are widely used as molecular markers in population genetic analysis (Bruford & Wayne, 1993; Vieira *et al.*, 2016). The first step of using microsatellite loci for population genetic studies is developing efficient and stable primers. Microsatellite markers are usually developed species-specifically to enhance PCR amplification efficacy and ensure high polymorphism (Kofler *et al.*, 2008; Song *et al.*, 2017; Gao *et al.*, 2019). For some closely related species, primers of microsatellite loci can be shared between species (Wanke *et al.*, 2006; Hai, 2009; Bello *et al.*, 2020). Microsatellites can be developed from genomic sequences and expressed sequence tags (EST). The microsatellites of ES have been identified from ESTs (Wu *et al.*, 2016). However, no high polymorphic microsatellite maker was developed for EB.

In this study, we characterized the distribution of microsatellites in genomes of EB and ES and developed microsatellite markers shared between these two species. We validated these markers in three populations of EB and ES. These novel and universal markers provide valuable tools for ecological, genetic, and evolutionary study of the two weevils.

2 Materials and methods

2.1 Sample collection and DNA extraction

Three populations of EB and ES were collected, respectively, from areas of China where these two species are causing increasing damages (Fig. 1). Adults of both species were sampled from their host of *A. altissima* from April to August of 2018. Thirteen adults of EB and ES were simultaneously collected from two locations of NXZW and BJHD. Twelve adults of each species were collected from SDTA (EB) and SXYL (ES). In total, 38 individuals of each species were used in our study. The samples were kept in absolute ethanol prior to DNA extraction. Total genomic DNA was extracted from the legs of each adult using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The voucher specimens were stored at -80°C in the Integrated Pest Management Laboratory, Beijing Academy of Agriculture

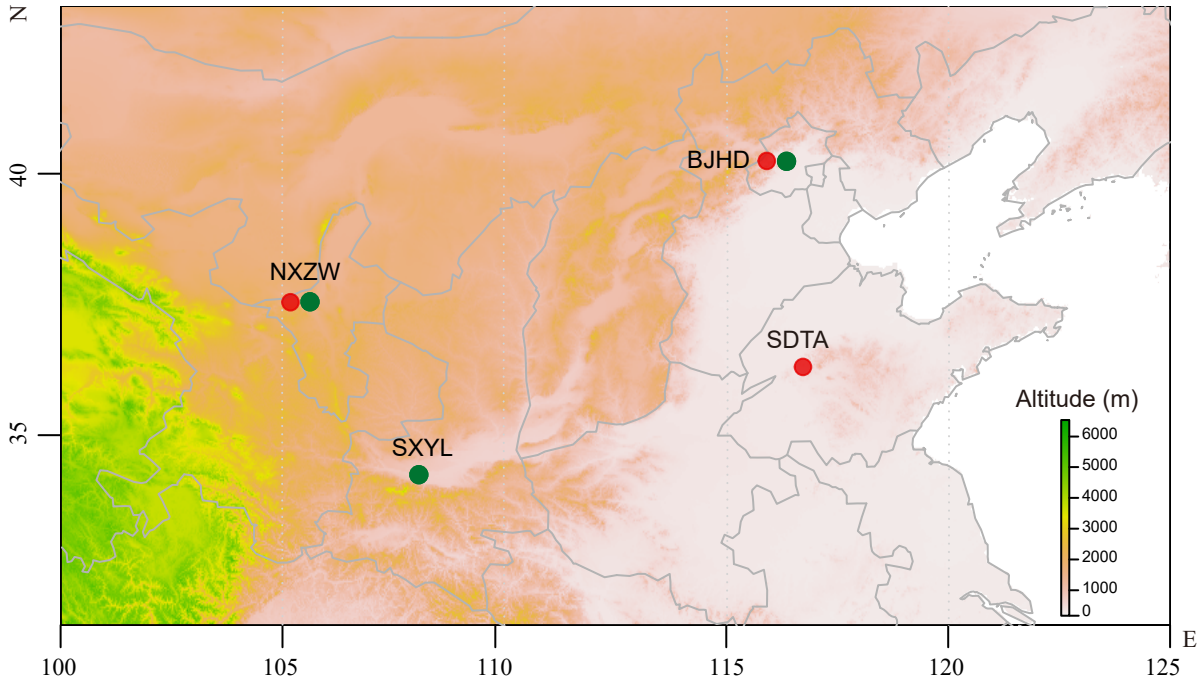

Figure 1. Collection sites for specimens of *Eucryptorrhynchus brandti* (red) and *E. scrobiculatus* (green). Abbreviations: BJHD—Haidian District, Beijing (116.22°E, 40.04°N); NXZW—Zhongwei, Ningxia (105.12°E, 37.50°N); SDTA—Tai'an, Shandong (116.72°E, 36.27°N); SXYL—Yangling, Shaanxi (108.07°E, 34.26°N).

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2.2 Characterizing the distribution of microsatellites in two weevils

The MSDB (http://msdb.biosv.com/) was used to identify potential microsatellite sites from the whole genome sequences of EB and ES (unpublished). A minimum of 250, 5, 5, 5, 5, and 5 repeats was used to distinguish mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide motif (Du *et al.*, 2013). We used a high value of 250 to eliminate microsatellites with mononucleotide repeat motifs. The distribution of microsatellite loci in the two genomes was summarized according to the criteria of previous studies (Cao *et al.*, 2017; Song *et al.*, 2017).

2.3 Development of universal microsatellite markers

The QDD (Meglecz *et al.*, 2010) was used to design the primers of microsatellite loci. First, we used the BLAST to search for the orthologous microsatellite regions between the two weevils. The BLAST hits with a similarity higher than 90% and the matching sequence length longer than 1000 bp were used as templates to design primers for these two weevils. The parameters of primer design were set as following: annealing temperature (Tm): 58°C < Tm < 62°C; the difference in Tm between paired upstream and downstream primers is < 4°C; only one pair of microsatellite primers is kept on each locus; the repeat motif of the amplified product is ≥3. The microsatellite loci were further filtered under stringent criteria as suggested in previous studies (Cao *et al.*, 2017; Song *et al.*, 2017): (i) the microsatellites had to be pure and specific, (ii) the design strategy of "A" in QDD was used, and (iii) the minimum distance between the 3′ end of a primer pair and its target region had to be no shorter than 10 bp.

We selected 60 pairs of primers for initial validation using twelve individuals of each species from BJHD of the two weevils. The amplified products were labeled using fluorescence following the method (Blacket *et al.*, 2012). Briefly, three fluorescences (FAM, ROX, HEX) were independently added to a primer C tail (PC-tail) (5′ CAGGACCAGGCTACCGTG 3′). The sequence of PC-tail was added to the 5' end of each upstream primer (Blacket *et al.*, 2012). The reaction system was set to 10 µl, including 0.5 µl of template DNA (5-20 ng/µl), 5 µl of Master Mix (Promega, Madison, WI, USA), 0.08 µl of PC tail modified forward primer (10 mM), 0.16 µl of reverse primer (10 mM), 0.32 µl of fluorescence-labeled PC-tail (10 mM) and 3.94 µl of ddH_2O. The following PCR amplification program was used to amplify the microsatellites: 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 56°C and 45 s at 72°C, followed by a final 10-min extension at 72°C.

Length of PCR products was analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, USA) using the GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems, USA) for sizing DNA fragments. Genotypes were determined using GENEMAPPER v4.0 (Applied Biosystems, USA) (Chatterji & Pachter, 2006).

2.4 Population genetic diversity analysis

The number of alleles, the observed heterozygosity (H_O), and the polymorphism information content (P_{IC}) were analyzed by the macros Microsatellite Tools. The null allele frequencies were estimated using the software FreeNA (Chapuis & Estoup, 2007). Deviation from Hardy-Weinberg equilibrium (HWE) for each locus/population combination, linkage disequilibrium (LD) among loci within each population, pairwise mean population differentiation (F_{ST}), and inbreeding coefficients (F_{IS}) was estimated in GENEPOP v4.0.11 (Rousset, 2008). We used the HP-RARE v1.1 (Kalinowski, 2005) to test allelic richness (A_R) and allelic richness of private alleles (P_{AR}) of each site. We used GENCLONE v2.0 (Arnaud-Haond & Belkhir, 2006) to estimate the total number of alleles (A_T) and the unbiased expected heterozygosity (H_E). We compared the number of alleles (A_S) and heterozygosity (H_{ES}) among samples with different sample sizes in GENCLONE (Arnaud-Haond & Belkhir, 2006).

2.5 Population genetic structure analysis

Population genetic structure was inferred using the STRUCTURE v2.3.4 program (Earl & Vonholdt, 2012). We used 30 replicates of each K value from 1 to 10, with 200,000 Markov chain Monte Carlo iterations and a burn-in of 100,000 iterations. Structure Harvester v0.6.94 was used to determine the optimal K value by a Delta K method (http://taylor0.biology.ucla.edu/structureHarvester/, last accessed on 12 August 2021). Membership coefficient matrices (Q-matrices) associated with the optimal K were processed using CLUMPP v1.12 (Jakobsson and Rosenberg, 2007), and then visualized using the DISTRUCT v1.1 (Taubert *et al.*, 2019).

3 Results and discussion

3.1 Characters and development of microsatellites in the two weevils

For the EB, a total of 12,524 potential microsatellites were identified. When we excluded loci with repeat motif of mononucleotide, there are 9,834 (24.47%) dinucleotide, 2,233 (5.56%) trinucleotide, 442 (1.1%) tetranucleotide, 11 (0.03%) pentanucleotide, 4 (0.01%) hexanucleotide microsatellites. The number of microsatellites with the five types of motif class decreased with the increase of motif length. Microsatellites with repeat AT, AC, AG, AAT, AAG motifs were the most common ones (Fig. 2). The microsatellites with the five most frequent repeat motifs occupy 90.80% of all microsatellites. The average length of the repeat regions with different repeat motif lengths ranges from 13.8 to 33 bp.

For the ES, a total of 11,030 potential microsatellites were identified. When we excluded loci with repeat motif of mononucleotide, there are 8,763 (27.53%) dinucleotide, 1,973 (6.20%) trinucleotide, 250 (0.79%) tetranucleotide, 23 (0.07%) pentanucleotide, 3 (0.01%) hexanucleotide microsatellites. The number of microsatellites with the five types of motif class decreased with the increase of motif length. Microsatellites with repeat AT, AC, AG, AAT, AAG motifs were the most common ones (Fig. 2). The microsatellites with the five most frequently repeat motifs occupy 91.34% of all microsatellites. The average length of the repeat regions with different repeat motif lengths ranges from 12.41 to 60 bp.

Our analysis showed that microsatellites are biased in AT-rich repeat motif in both EB and ES, which was reported in other species of Coleoptera, such as *Austroplatypus incompertus* (Platypodinae) (Coleoptera: Curculionidae) (Smith *et al.*, 2011), *Tomicus yunnanensis* (Scolytinae) (Coleoptera: Scolytinae) (Yuan *et al.*, 2014) and *Octodonta nipae* (Maulik) (Coleoptera: Chrysomelidae) (Chen *et al.*, 2018). However, in some species of Thysanoptera and Lepidoptera, the pattern of repeat motif usage is different. For example, in the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae), microsatellites are biased in the AG-rich repeat motif, and the fall webworm *Hyphantria cunea* (Lepidoptera: Arctiidae) (Cao *et al.*, 2015), microsatellites are biased in AC-rich repeat motif. In EB and ES, microsatellites with dinucleotide repeat motifs are the most common ones, which is congruent with the melon thrips *Thrips palmi* (Thysanoptera: Thripidae) and the oriental fruit moth *Grapholita molesta* (Lepidoptera: Tortricidae), all of which used genome sequence for microsatellite development (Song *et al.*, 2017; Gao *et al.*, 2019). Trinucleotide microsatellite is the most common in ESTs of ES (Wu *et al.*, 2016), while dinucleotide microsatellite is the most common in our genome-wide analysis.

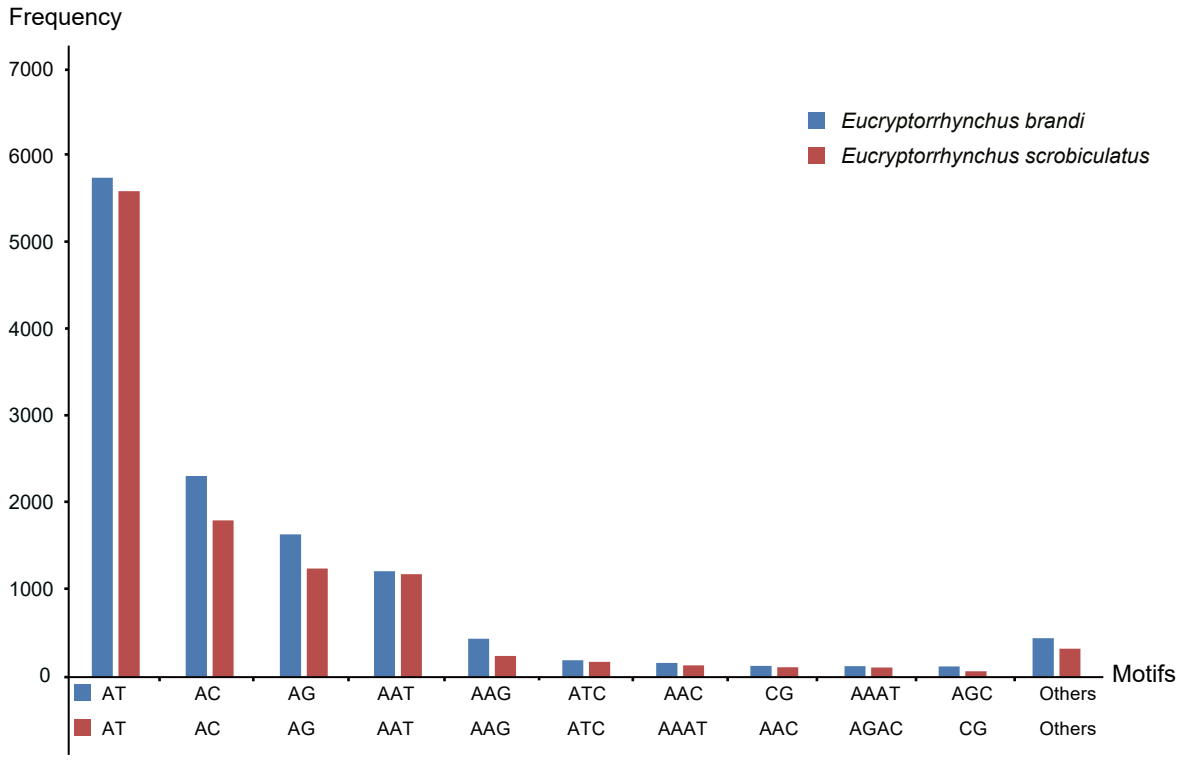

Figure 2. Frequency distribution of microsatellites among different motifs in the *Eucryptorrhynchus brandti* and *E. scrobiculatus*. The "others" category represents summed motifs with counts below 100.

Thirty-eight loci with an amplification rate higher than 75% in 24 testing individuals were remained out of 60 loci after initial testing. In the validation of the 38 loci using four populations, we discarded loci with an amplification rate lower than 75% and allele number less than two in three populations. We kept 14 loci for subsequent analysis of both species (Table 1).

Table 1. Primer sequence and characters of microsatellite loci developed for two weevils of *Eucryptorrhynchus brandti* and *E. scrobiculatus*.

Locus	Dye	Forward primer sequence (5'– 3')	Reverse primer sequence (5'– 3')	Motifs	Size (bp)	Ta* (°C)
S01	HEX	GGTGGAACATCACGCAAACA	TGCGCGTTGATTATCTTTCAGA	$(AT)_6$	95	56
S07	HEX	AGGCGTTATTAGTGGGTGGA	CATCCCTGTGTTGTGCTTCC	$(AG)_7$	140	56
S09	HEX	GTATTAGTGGCAACGTCGGATT	CTTCGCGGCGAGCCAAAG	$(AT)_5$	140	56
S10	HEX	AAGACCAGATGAAGAATTCCTTGG	TCATTCTCATCAGGTATTTCTGGT	$(AT)_5$	141	56
S12	HEX	CTCCAAGCGCTCGATGTCTT	CGACAGCTCGGAAATGATCG	$(AAG)_7$	142	56
S18	HEX	CAAGACACGCCTTGGAAACA	ATCCGAAGTCAGAGTGGTCG	$(AT)_5$	173	56
S19	HEX	CCGAGGCTTCTGTTAACGTC	TCCTGTACCTCGTTACGTCA	$(AG)_5$	179	56
S21	FAM	CCAAAGGAGGATGTTCAACAT	GGAATTAATGTGCGCGGCT	$(AC)_7$	188	56
S27	FAM	GGATACACGATTCCTATCCAGT	CGATAGGTTCCAGTTGCAGG	$(AAAT)_5$	191	56
S38	FAM	TGGACTACAGTGTCAAGGCG	AGGACTGGTTGATGGGAGTC	$(AC)_{11}$	243	56
S40	FAM	CGGAGCTCAAGGTAATCGAC	GAGACGGATTTCTCAAGGGC	$(AT)_5$	251	56
S54	ROX	CCCTGTTCAAGCCACCCTAA	CTTCGGGTTAGCCGTCGAA	$(AGCC)_6$	342	56
S59	ROX	GTACTCGAAACGTGACTCGC	ACCATTCAAGGAGGACGGG	$(AC)_5$	348	56
S60	ROX	AAACCTGACACAGAGGAGGC	GGAGGATCGACGCCTTTAGA	$(AT)_5$	349	56

*Ta—annealing temperature for PCR amplification.

3.2 Population genetic diversity

No locus deviated from Hardy-Weinberg equilibrium in all populations of EB and ES. In EB, S09 and S38 showed linkage disequilibrium in the NXZW population, while in ES, linkage disequilibrium was found in S01 and S18, S07 and S27/S40, S10 and S40, S27 and S40 in BJHD population (Table 2). The linkage of loci in some populations might be caused by the collection of siblings (Song *et al.*, 2017). The polymorphic information content (PIC) value ranged from 0.22–0.24 in EB and 0.11–0.19 in ES. These two weevils have lower PIC values compared with *Hyphantria cunea* (Lepidoptera: Arctiidae) (Cao *et al.*, 2015) and *Anoplophora glabripennis* (Coleoptera: Cerambycidae) (Liu *et al.*, 2017).

In EB, the observed heterozygosity (H_O) for each population ranges from 0.17 and 0.24; the inbreeding coefficient (F_{IS}) ranges from 0.02 to 0.42; the allelic richness (A_R) of three populations were not significantly different, varying from 2.07 in SDTA to 2.40 in BJHD. The private allelic richness was the lowest in the SDTA population. The standardized total number of alleles (A_S) ranged from 57.59 in NXZW to 67.88 in BJHD. The standardized expected heterozygosity (H_{ES}) varies from 0.44 in SDTA to 0.54 in NXZW (Table 3). The population differentiation coefficient (F_{ST}) ranges from 0.19 (NXZW *vs* SDTA) to 0.33 (BJHD *vs* SDTA).

In ES, the observed heterozygosity (H_O) for each population ranges from 0.06 to 0.18; the inbreeding coefficient (F_{IS}) ranges from -0.04 to 0.42; the allelic richness (A_R) of three populations were not significantly different, varying from 1.52 in SXYL to 1.85 in BJHD. The private allelic richness was the lowest in the NXZW population. The standardized total number of alleles (A_S) ranged from 27.18 in NXZW to 30.20 in BJHD. The standardized expected heterozygosity (H_{ES}) varies from 0.17 in NXZW to 0.27 in SXYL (Table 3). The F_{ST} ranges from 0.54 (BJHD *vs* NXZW) to 0.57 (BJHD *vs* SXYL).

3.3 Population genetic structure

Structure analysis showed that the optimal K is three in both species. Each population is mainly composed of a major genetic cluster (Fig. 3). We identified admixtures in populations of the two weevils. In EB, two individuals of BJHD population and one individual of NXZW population have a high proportion of genetic cluster mainly distributed in SDTA (yellow in Fig. 3a). In ES, one individual in BJHD has a high proportion of genetic cluster mainly distributed in SXYL (green in Fig. 3b), while one individual in NXZW has a high proportion of genetic cluster mainly distributed in BJHD (yellow in Fig. 3b).

Based on pairwise population F_{ST} and STRUCTURE analysis, our analysis showed a relatively high level of population differentiation in both EB and ES. The underlying ecological and evolutionary mechanism shaping the population differentiation of these two weevils needs further exploration.

Table 2. Hardy-Weinberg equilibrium (HWE) and null allele frequency of 14 microsatellite loci in three populations of *Eucryptorrhynchus brandti* and *E. scrobiculatus*.*

Species		HWE			Null allele frequency		
	Locus	BJHD	NXZW	SDTA	BJHD	NXZW	SDTA
E. brandti	S01	NA	NA	NA	0.000	0.001	0.001
	S07	NA	NA	NA	0.001	0.001	0.001
	S09	0.406	0.578	0.354	0.071	0.000	0.013
	S10	0.001	0.040	NA	0.288	0.182	0.000
	S12	NA	0.000	NA	0.001	0.329	0.001
	S18	0.964	0.285	1.000	0.000	0.013	0.000
	S19	0.040	NA	NA	0.182	0.001	0.001
	S21	0.682	NA	1.000	0.000	0.001	0.000
	S27	0.374	0.598	0.560	0.000	0.000	0.000
	S38	0.040	1.000	1.000	0.182	0.000	0.000
	S40	NA	0.002	NA	0.001	0.256	0.001
	S54	1.000	NA	0.404	0.000	0.000	0.050
	S59	0.001	0.038	0.001	0.232	0.214	0.332
	S60	NA	0.155	NA	0.001	0.103	0.001
E. scrobiculatus	S01	1.000	0.040	NA	0.000	0.182	0.000
	S07	0.256	0.001	1.000	0.115	0.283	0.000
	S09	0.040	NA	NA	0.182	0.001	0.001
	S10	0.773	1.000	0.092	0.000	0.000	0.000
	S12	1.000	0.040	NA	0.000	0.182	0.000
	S18	0.602	NA	0.594	0.000	0.001	0.054
	S19	0.040	0.005	NA	0.182	0.300	0.001
	S21	NA	1.000	NA	0.001	0.000	0.000
	S27	0.039	NA	NA	0.166	0.001	0.001
	S38	0.040	0.044	0.046	0.182	0.188	0.000
	S40	0.001	0.040	NA	0.254	0.182	0.001
	S54	NA	NA	NA	0.001	0.001	0.001
	S59	NA	NA	NA	0.182	0.182	0.001
	S60	NA	NA	NA	0.182	0.182	0.001

*NA—not available.

Table 3. Parameters of genetic diversity in populations of *Eucryptorrhynchus brandti* and *E. scrobiculatus*.*

Species	Population	N	A_T	A_S	A_R	P_{AR}	H_O	H_{ET}	H_{ES}	F_{IS}
E. brandti	BJHD	13	71	67.88	2.40	0.52	0.21	0.50	0.50	0.33
	NXZW	13	59	57.59	2.18	0.14	0.17	0.54	0.54	0.42
	SDTA	12	61	61.00	2.07	0.03	0.24	0.44	0.44	0.02
E. scrobiculatus	BJHD	13	31	30.20	1.85	0.30	0.17	0.26	0.26	0.01
	NXZW	13	28	27.18	1.55	0.07	0.06	0.18	0.17	0.42
	SXYL	12	29	29.00	1.52	0.12	0.18	0.27	0.27	-0.04

*Abbreviations: N—Sample size; A_R—average allelic richness; P_{AR}—private allelic richness; A_T—total number of alleles; A_S—standardized total number of alleles; H_O—observed heterozygosity; H_{ET}—expected heterozygosity; H_{ES}—standardized expected heterozygosity; F_{IS}—inbreeding coefficient.

4 Conclusion

In our study, we characterized the distribution patterns of microsatellites in genomes of EB and ES. We developed 14 highly polymorphic microsatellite markers for both species. Based on these molecular markers, we found a high genetic diversity within three testing populations and high genetic differentiation among these populations. These markers provide valuable tools for the comparative study of these two weevils. Further studies from ecological, genetic, and evolutionary aspects using the developed markers may shed light on our understanding of the outbreak of the two weevils.

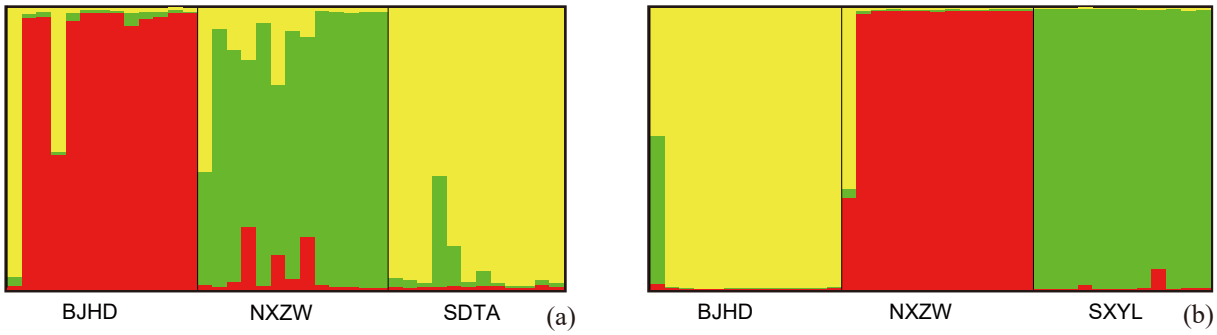

Figure 3. Genetic structure of *Eucryptorrhynchus brandti* (a) and *E. scrobiculatus* (b) populations based on 14 microsatellite markers inferred using the software STRUCTURE. Each bin indicates an individual. Different colors show the identified clusters. The best number of clusters (K) is 3. Abbreviations: BJHD—Haidian District, Beijing; NXZW—Zhongwei, Ningxia; SDTA—Tai'an, Shandong; SXYL—Yangling, Shaanxi.

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