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Genetic diversity and phylogenetic relationships among and within populations of *Whitmania pigra* and *Hirudo nipponica* based on ISSR and SRAP markers

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ABSTRACT

The objective of this study was to obtain an overview of the genetic relationships among and within *Whitmania pigra* and *Hirudo nipponica* using the ISSR and SRAP markers that were derived from related accessions, 11 *W. pigra* and 4 *H. nipponica*, from 8 provinces in China. All populations were uniquely fingerprinted by two markers. Mean genetic similarities were estimated at 0.76 and 0.79 using the ISSRs and SRAPs. Two main clusters that separated the *W. pigra* and *H. nipponica* populations were produced using UPGMA analysis. The Mantel test resulted in a good fit (SRAP = 0.80609) to the fit (ISSR = 0.76006) of the cophenetic values. Comparing the two marker systems, the ISSR and SRAP similarity indices were correlated ($r = 0.8564$). Finally, an appropriate strategy for conserving the *Hirudo* germplasm is proposed.

1. Introduction

Hirudo live naturally in moist places on the aquatic plants or algae of many rivers and lakes. Their geographical distribution is throughout the world; however, *Whitmania pigra* and *Hirudo nipponica* are Chinese species that have commonly been known as traditional Chinese anticoagulant medicines for thousands of years, including *W. pigra* Whitman, *H. nipponica* Whitman and *Whitmania acranulata* Whitman; in clinical practice, these species have been used for promoting blood circulation and stasis relief (Chinese Pharmacopoeia Editorial Committee, 2010). As an anticoagulant medicine, they are also widely applied in the United States and Europe. It is currently believed that cardiovascular disease, a serious threat to human health (Ozono, 2006), could be cured by proprietary Chinese traditional medicines and health products using *Hirudo* as a major component. *H. nipponica* (Nikonov et al., 1999), *W. pigra* (Jin and Zhang, 2002; Shen et al., 2002; Shi et al., 2006, 2007; Guo et al., 2006; Liu et al., 2010, 2011), *Hirudinaria manillensis* (Zhang et al., 2008) and *Hirudo medicinalis* (Kasparek et al., 2000) have been studied extensively for decades; however, knowledge of their phylogenetic relationships and of the distribution and status between and within *Hirudo* is currently limited.

Molecular markers could be an appropriate tool for identifying the species. Moreover, molecular markers are effective for DNA fingerprinting, genetic diversity analyses and germplasm evaluation. Sequence-related amplified polymorphism (SRAP)

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(Li and Quiros, 2001) has been recognised as a new and useful molecular marker system in buffalograss (Budak et al., 2004) and medicinal *Chrysanthemum morifolium* (Shao et al., 2010). The genetic characteristics of *W. pigra* and *H. nipponica* are currently largely unknown.

W. pigra and *H. nipponica* are breeds of *Hirudo* that are listed in the <The Pharmacopoeia of the People's Republic of China> (Chinese Pharmacopoeia Editorial Committee, 2010). The efficacies of the two species are similar, but they belong to *Haemopidae*, *Whitmania* and *Hirudinidae*, *Hirudo*, respectively. To the best of such knowledge, no study has examined the application of ISSR or SRAP markers to the genetic diversity of the *W. pigra* and *H. nipponica* programme. The present study was conducted to understand the genetic diversity and genetic relationships of the various accessions sampled from 15 representative populations (225 individuals) using ISSR and SRAP markers. The objectives are to (1) more clearly define the genetic similarity among and within populations of *W. pigra* and *H. nipponica* in China and (2) compare different marker techniques. This study will aid in the long-term objective of identifying genetic diversity analyses among and within populations of *W. pigra* and *H. nipponica* using molecular markers.

2. Materials and methods

2.1. Animal materials

The animal materials used in this investigation were from 15 populations and represented almost all of the natural distribution areas of *Hirudo* in China. These populations can be grouped into two species, i.e., *W. pigra* and *H. nipponica*. The population from the three regions, i.e., South China (GL, GZ, LA, DL), East China (SY, JH, DF, TXC, TXW, JR, SQ, NJC, MAS, LY) and North China (HS), includes two artificial breeding varieties (NJC, TXC) and the remainder of the population contains wild species (Fig. 1 and Table 1). A total of 225 individuals from the 15 populations were included in this study. Fresh abdominal muscle from each animal was collected and immediately dried with silica gel. All samples were stored at −70 °C until processing.

2.2. DNA extraction, primers, PCR conditions, and gel electrophoresis

Total genomic DNA was extracted using the protocol established by Sambrook and Russell (2001). The quality and quantity of the DNA were determined using 0.8% agarose gel electrophoresis. DNA samples were diluted to 20 ng/ml with 1× TE buffer and stored at −20 °C prior to ISSR and SRAP analyses.

The ISSR and SRAP primers employed are listed in Table 2 [Invitrogen Biotech (Shanghai) Co., China]. For all methods, the polymerase chain reaction (PCR) mixtures and electrophoresis conditions were performed as described by Budak et al. (2004). Amplifications were performed using a thermocycler PTC 200™ Programmable Thermal Controller (Bio-Rad, USA) for one cycle of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 50 °C for the ISSR analysis; and 1 min at 72 °C, followed by a final extension step at 72 °C for 5 min before cooling the reaction mixture to 10 °C. Based on the expected numbers and the sizes of the restriction fragments that were visualised on the 2.5% agarose gels stained with ethidium bromide, ten microlitres

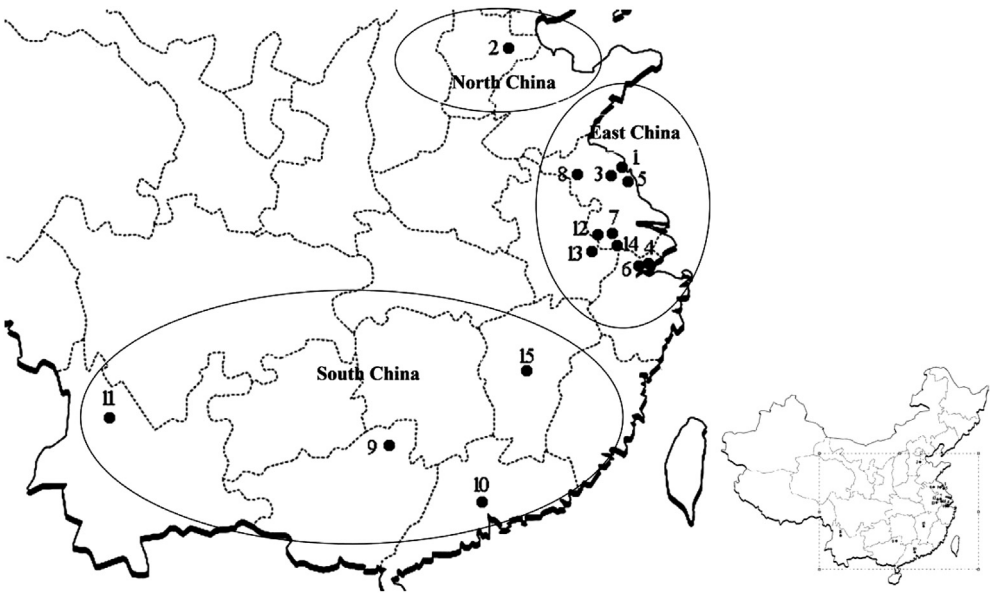

Fig. 1. The locations of populations sampled in this study were assigned to three geographical regions as described in Table 1.

Table 1
The *W. pigra* and *H. nipponica* populations used in the present study.

Population code	Individual code	Sample size	Location	Scientific name	Longitude (E)	Latitude (N)
SY	1–15	15	Sheyang, Jiangsu Province	*Whitmania pigra*	120.18	33.49
HS	16–30	15	Hengshui, Hebei Province	*Whitmania pigra*	115.35	37.33
JH	31–45	15	Jianhu, Jiangsu Province	*Whitmania pigra*	119.47	33.27
TXW	46–60	15	Tongxiang wild, Zhejiang Province	*Whitmania pigra*	120.23	30.38
DF	61–75	15	Dafeng, Jiangsu Province	*Whitmania pigra*	120.24	33.12
TXC	76–90	15	Tongxiang cultivated, Zhejiang Province	*Whitmania pigra*	120.33	30.35
JR	91–105	15	Jvrong, Jiangsu Province	*Whitmania pigra*	119.12	31.52
SQ	106–120	15	Suqian, Jiangsu Province	*Whitmania pigra*	118.12	33.27
GL	121–135	15	Guilin, Guangxi Province	*Hirudo nipponica*	109.56	25.47
GZ	136–150	15	Guangzhou, Guangdong Province	*Hirudo nipponica*	113.13	23.06
DL	151–165	15	Dali,Yunnan Province	*Hirudo nipponica*	100.16	25.37
NJC	166–180	15	Nanjing cultivated, Jiangsu Province	*Whitmania pigra*	118.50	32.02
MAS	181–195	15	Maanshan, Anhui Province	*Hirudo nipponica*	118.50	32.02
LY	196–210	15	Liyang, Jiangsu Province	*Whitmania pigra*	119.22	31.23
LA	211–225	15	Le'an, Jiangxi Province	*Whitmania pigra*	115.46	27.16

Table 2
The primers used for the molecular analysis.

Primer	Sequence	Primer	Sequence
(a) ISSR			
ISSR-3	ACACACACACACACACTT	ISSR-48	TGTGTGTGTGTGTGTGAA
ISSR-4	ACACACACACACACACAG	ISSR-49	TGTGTGTGTGTGTGTGAC
ISSR-5	ACACACACACACACACTG	ISSR-50	TGTGTGTGTGTGTGTGAG
ISSR-9	CTCCTCCTCCTCCTCCTC	ISSR-55	TGTGTGTGTGTGTGTGGG
ISSR-10	GAAGAAGAAGAAGAAGAA	ISSR-56	AGAGAGAGAGAGAGAGTT
ISSR-12	GAGGAGGAGGAGGAGGAG	ISSR-57	AGAGAGAGAGAGAGAGTG
ISSR-17	GACAGACAGACAGACA	ISSR-58	AGAGAGAGAGAGAGAGGA
ISSR-22	ACACACACACACACACAA	ISSR-60	AGAGAGAGAGAGAGAGGG
ISSR-24	ACACACACACACACACTC	ISSR-61	AGAGAGAGAGAGAGAGGT
ISSR-26	ACACACACACACACACCC	ISSR-64	AGAGAGAGAGAGAGAGCG
ISSR-27	TGTGTGTGTGTGTGTGCG	ISSR-65	AGAGAGAGAGAGAGAGCC
ISSR-28	TGTGTGTGTGTGTGTGCC	ISSR-67	TCTCTCTCTCTCTCTCCC
ISSR-30	TGTGTGTGTGTGTGTGTC	ISSR-68	TCTCTCTCTCTCTCAG
ISSR-33	AGAGAGAGAGAGAGAGAT	ISSR-69	TCTCTCTCTCTCTCTG
ISSR-34	AGAGAGAGAGAGAGAGAA	ISSR-70	TCTCTCTCTCTCTCGA
ISSR-35	AGAGAGAGAGAGAGAGTA	ISSR-77	ACTCACTCACTCACTC
ISSR-40	ACCACCACCACCACCACC	ISSR-Y1	GACAGACAGACAGACA
ISSR-43	ACACACACACACACACCT	ISSR-Y2	BDBCACACACACACA
ISSR-44	ACACACACACACACACGA	ISSR-Y11	GAGAGAGAGAGAGAGAT
ISSR-45	ACACACACACACACACGC		
(b) SRAP			
E1	GACTGCGTACGAATTCAA	M1	TGAGTCCAAACCGGATA
E2	GACTGCGTACGAATTCTG	M2	TGAGTCCAAACCGGAGC
E3	GACTGCGTACGAATTGAC	M3	TGAGTCCAAACCGGACC
E4	GACTGCGTACGAATTTGA	M4	TGAGTCCAAACCGGACA
E5	GACTGCGTACGAATTAAC	M5	TGAGTCCAAACCGGTGC
E6	GACTGCGTACGAATTGCA	M6	TGAGTCCAAACCGGAGA
E7	GACTGCGTACGAATTGAG	M7	TGAGTCCAAACCGGACG
E8	GACTGCGTACGAATTGCC	M8	TGAGTCCAAACCGGAAA
E9	GACTGCGTACGAATTTCA	M9	TGAGTCCAAACCGGAAC
E10	GACTGCGTACGAATTCAT		

	M1	M2	M3	M4	M5	M6	M7	M8	M9
(c) Polymorphism of different SRAP primer combination									
E1	+	–	+	+	+	–	–	–	–
E2	–	–	–	+	–	–	–	+	–
E3	–	–	+	–	+	+	–	+	+
E4	–	–	+	+	–	–	+	–	–
E5	+	+	–	+	–	+	–	+	+
E6	+	+	+	–	+	+	–	+	+
E7	–	+	–	–	+	+	+	+	–
E8	–	–	–	–	+	–	–	–	–
E9	–	–	–	–	+	–	–	+	–
E10	+	–	–	–	+	–	–	+	+

(a) For the ISSR analysis; (b) For the SRAP analysis; (c) Polymorphisms of the different SRAP primer combinations. Horizontal lines are forward primers; vertical row are reverse primers. –: Monomorphism, +: Polymorphism.

of PCR products were used. Amplified fragments (5 cycles of 1 min at 94 °C, 1 min at 35 °C and 1 min at 72 °C; 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C; 1 cycle of 4 min; and one cycle of 4 min at 10 °C for the SRAPs) were photographed under ultraviolet light using a Bio-Imaging System (Syngene, Genegenuis). The PCR products (25 ml) were fractionated using 12% polyacrylamide gel electrophoresis and a Hoefer vertical-gel apparatus (SE600) (Bassam et al., 1991). The amplifications were repeated twice, and only clear repetitive bands were used for the data analysis. Molecular weights were estimated using a DNA marker (DL2000; Takara Biotech Co., Ltd).

2.3. Scoring gels, data analysis and combined data analyses

Popgene version 3.2 (Yeh et al., 1999), AMOVA (Excoffier et al., 1992) and the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 software package (Rohlf, 2004) were used to calculate the parameters for the genetic diversity as follows: (1) PPB (percentage of the polymorphic band); (2) Ao (observed number of alleles per locus) and Ae (effective number of alleles per locus); (3) He (Nei's gene diversity) and I (Shannon's information index); (4) Nei's genetic distances (D) and genetic identity (IN) (Dice, 1945; Nei and Li, 1979); (5) cluster analysis with unweighted pair group with arithmetic average (UPGMA) (Sun et al., 2002); (6) coefficient of gene differentiation among populations within species (Gst) (Nei, 1972); (7) gene flow (Nm); and (8) Mantel test between geographic and Nei's genetic distance (Mantel, 1967).

A binary matrix was obtained by visually scoring the bands for both of the ISSR and SRAP markers. Amplifications were repeated once for each primer, and only consistent bands were considered in further analyses. The efficiency of discrimination was assessed in terms of the number of polymorphic markers generated and the ability to generate unique genotypes. Marker utility was a function of the information content per marker and the number of markers generated per assay, also known as the effective multiplex ratio (EMR). Marker utility has previously been estimated thus: marker index, MI = average expected heterozygosity (Hav) × EMR (Powell et al., 1996; Milbourne et al., 1997). However, estimating the expected heterozygosity might not be appropriate for crossbreeding heterozygous populations that are analysed by multilocus dominant markers (McGregor et al., 2000). The information content of a marker can also been expressed in terms of band informativeness (Prevost and Wilkinson, 1999). In the absence of primers and probes providing co-dominant markers in *Hirudo* and the lack of statistical techniques for the unambiguous treatment of multilocus marker data to calculate heterozygosity, we have opted for the MI modification. Instead of averaging the expected heterozygosity (Hav), an index known as the average band informativeness (Ibav) was employed to estimate the information content per marker. The average band informativeness of a technique, with a range from 0 to 1, was calculated as

$$Ibav = \frac{1}{n}\sum_{i-1}^{n} 1 - (2|0.5 - p_i|)$$

where p_i is the proportion of accessions containing the ith amplicon and n is the total number of amplicons. Thus, marker utility was estimated as MI = Ibav × EMR.

3. Results

3.1. Polymorphism level

Thirty-nine ISSR primers and thirty-nine of the 90 SRAP primers (Table 2) were used to estimate the genetic relationships among and within the *W. pigra* and *H nipponica* biotypes.

In the ISSRs, 456 bands were observed in total, 454 of which (99.56%) were polymorphic among the *W. pigra* and *H. nipponica* biotypes and were shared between at least two individuals. Table 3 shows the average genetic diversity and PPB for each population, with a total average genetic diversity of 0.3490. The "GL" population had the highest PPB (53.95%) and the "TXC" population had the lowest (29.17%). The Ae was also lower than the Ao, ranging from 1.1558 to 1.3054. Shannon's information index (I) ranged from 0.1352 to 0.3085, with the same trend line as PPB and He. The genetic variation indices at the species' level were PPB = 99.56%, Ao = 1.9956, Ae = 1.5966, He = 0.3490 and I = 0.5219 (Table 3).

In total, 503 of the 508 bands (99.02%) were polymorphic among the *W. pigra* and *H. nipponica* biotypes in the SRAPs. The average genetic diversity and percentage of polymorphic bands for each population are summarised in Table 3. The "GL" population had the highest PPB (56.06%) and the "NJC" population had the lowest (31.69%). The Ae ranged from 1.2913 to 1.4134 and was some what higher than the Ao. Shannon's information index (I) ranged from 0.1785 to 0.3374 and showed the same trend line that was observed for the PPB and He. The genetic variations indicated at the species level were PPB = 99.02%, Ao = 1.9902, Ae = 1.6495, He = 0.3687 and I = 0.5433 (Table 3).

3.2. Level of diversity and molecular variance

Based on the values for Nei's total gene diversity (Ht = 0.3490) and Nei's gene diversity within populations (Hs = 0.1490), Nei's genetic differentiation (Gst) was calculated to be 0.5730 in the ISSRs using the Popgene software, whereas Ht = 0.3717, Hs = 0.1969 and Gst = 0.4699 for the SRAPs. Therefore, a relatively higher level of genetic differentiation within the

Table 3
Genetic variation of the fifteen populations of *W. pigra* and *H. nipponica*.

Population	PPB (%)	Ao		Ae		He		I	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
(a) ISSR									
SY	37.28	1.3728	0.4841	1.2933	0.4004	0.1611	0.2140	0.2316	0.3048
HS	34.65	1.3465	0.4764	1.2536	0.3805	0.1411	0.2036	0.2050	0.2907
JH	38.38	1.3838	0.4868	1.2924	0.3979	0.1615	0.2122	0.2333	0.3025
TXW	35.75	1.3575	0.4798	1.2673	0.3824	0.1493	0.2063	0.2163	0.2954
DF	36.84	1.3684	0.4829	1.2819	0.3896	0.1566	0.2102	0.2260	0.3006
TXC	29.17	1.2917	0.4550	1.2187	0.3612	0.1220	0.1956	0.1767	0.2802
JR	39.25	1.3925	0.4889	1.3054	0.4050	0.1676	0.2156	0.2412	0.3067
SQ	35.75	1.3575	0.4798	1.2692	0.3860	0.1495	0.2075	0.2163	0.2966
GL	53.95	1.5395	0.4990	1.3776	0.4085	0.2111	0.2149	0.3085	0.3044
GZ	46.49	1.4649	0.4993	1.3184	0.3936	0.1795	0.2100	0.2632	0.2994
DL	41.23	1.4123	0.4928	1.2426	0.3493	0.1422	0.1911	0.2132	0.2760
NJC	25.00	1.2500	0.4335	1.1558	0.3063	0.0907	0.1685	0.1352	0.2448
MAS	42.32	1.4232	0.4946	1.2652	0.3704	0.1519	0.1993	0.2254	0.2853
LY	32.68	1.3268	0.4695	1.1851	0.3204	0.1092	0.1760	0.1649	0.2557
LA	37.28	1.3728	0.4841	1.2488	0.3635	0.1424	0.1976	0.2098	0.2844
Mean	37.73	1.3773	0.4804	1.2650	0.3743	0.1490	0.2015	0.2178	0.2885
Species level	99.56	1.9956	0.0662	1.5966	0.2999	0.3490	0.1337	0.5219	0.1635
(b) SRAP									
SY	53.74	1.5374	0.4991	1.3559	0.3981	0.3981	0.2117	0.2955	0.3003
HS	45.87	1.4587	0.4988	1.3251	0.4026	0.4026	0.2144	0.2630	0.3045
JH	50.20	1.5020	0.5005	1.3578	0.4099	0.1987	0.2167	0.2891	0.3072
TXW	50.39	1.5039	0.5005	1.3501	0.4030	0.1961	0.2139	0.2866	0.3038
DF	51.18	1.5118	0.5004	1.3686	0.4089	0.2050	0.2174	0.2978	0.3086
TXC	42.13	1.4213	0.4942	1.2913	0.3867	0.1636	0.2086	0.2390	0.2977
JR	50.00	1.5000	0.5005	1.3511	0.4058	0.1958	0.2152	0.2856	0.3054
SQ	47.24	1.4724	0.4997	1.3287	0.4034	0.1829	0.2143	0.2667	0.3040
GL	58.46	1.5846	0.4933	1.4150	0.4120	0.2311	0.2160	0.3368	0.3050
GZ	53.94	1.5394	0.4989	1.3695	0.4130	0.2052	0.2163	0.2999	0.3050
DL	52.17	1.5217	0.5000	1.3527	0.4036	0.1978	0.2132	0.2900	0.3019
NJC	31.69	1.3169	0.4657	1.2206	0.3666	0.3666	0.1957	0.1785	0.2786
MAS	56.30	1.5630	0.4965	1.3957	0.4021	0.2228	0.2133	0.3261	0.3022
LY	56.69	1.5669	0.4960	1.3919	0.4036	0.2202	0.2141	0.3218	0.3034
LA	58.86	1.5886	0.4926	1.4134	0.4058	0.2314	0.2150	0.3374	0.3044
Mean	50.59	1.5059	0.4958	1.2954	0.4017	0.2412	0.2131	0.2876	0.3021
Species level	99.02	1.9902	0.0988	1.6495	0.3073	0.3687	0.1369	0.5433	0.1688

(a) Based on the RAPD analysis; (b) Based on the ISSR analysis; (c) Based on the SRAP analysis. PPB: Percentage of polymorphic bands; Ao: Observed number of alleles per locus; Ae: Effective number of alleles per locus; He: Nei's gene diversity; I: Shannon's information index.

populations was indicated by the ISSRs; however, the Gst = 0.4699 in the SRAPs, indicating that 47% of the gene differentiation occurred among populations and 53% occurred within populations. These results indicate a relatively lower level of genetic differentiation within the populations. The average numbers of individuals exchanged between populations per generation (Nm) (the number of migrating individuals deduced from the gene differentiation coefficient) were 0.3727 and 0.5641 by the ISSRs and SRAPs.

3.3. Phylogenetic analysis

The genetic similarities among all populations ranged from 0.63 to 0.89, with a mean similarity of 0.76, in the ISSRs; 0.67 to 0.91, with an mean similarity of 0.79, in the SRAPs; and a pairwise genetic distance between 0.10 and 0.40, in the SRAPs (Table 4). Based on the UPGMA analysis, the fifteen populations were grouped into 5 clusters using the ISSRs and into 6 clusters using the SRAPs (Fig. 2). The results of the PCA analysis were comparable to those obtained using the cluster analysis. The three most informative PC components explained 41.6% and 43.1% of the total variation by the ISSRs and SRAPs, respectively. The Mantel test was conducted to clarify the relationships among the populations. The matrix correlation (r) was 0.76006, corresponding to a fit among the populations in the ISSRs and the matrix correlation (r) was 0.80609, corresponding to a good fit among the populations in the SRAPs. The highest genetic identity was between "DF" and "TXC", whereas the lowest was found between "MAS" and "NJC" in the ISSRs. In the SRAPs, among the 15 populations, both the genetic similarities and the pairwise genetic distances were similar to the ISSRs. The genetic identity between "DF" and "SY" was the highest, whereas the identity between "MAS" and "LY" was the lowest. The Mantel test was performed between Nei's genetic and pairwise geographic distances.

Table 4
Genetic identities and distances of the fifteen *W. pigra* and *H. nipponica* populations.

ID	SY	HS	JH	TXW	DF	TXC	JR	SQ	GL	GZ	DL	NJC	MAS	LY	LA
(a) ISSR															
SY	–	0.7987	0.8441	0.8107	0.8504	0.8494	0.8198	0.8137	0.6877	0.6964	0.6887	0.7765	0.6871	0.7960	0.7581
HS	0.2247	–	0.7786	0.7987	0.7759	0.7880	0.7841	0.7788	0.6653	0.6802	0.6818	0.7102	0.6800	0.7529	0.7347
JH	0.1694	0.2502	–	0.8237	0.8395	0.8369	0.8284	0.8163	0.7063	0.6925	0.7045	0.7450	0.6968	0.7784	0.7462
TXW	0.2099	0.2248	0.1940	–	0.8683	0.8339	0.8383	0.8120	0.6473	0.6824	0.6989	0.7580	0.6490	0.7703	0.7369
DF	0.1621	0.2538	0.1750	0.1412	–	0.8883	0.8722	0.8331	0.6772	0.7019	0.7100	0.7712	0.6612	0.8031	0.7714
TXC	0.1632	0.2382	0.1780	0.1817	0.1184	–	0.8847	0.8315	0.6864	0.6947	0.6979	0.7568	0.6819	0.8149	0.7888
JR	0.1987	0.2433	0.1883	0.1764	0.1367	0.1225	–	0.8305	0.6772	0.6860	0.6993	0.7491	0.6613	0.8001	0.8213
SQ	0.2061	0.2500	0.2029	0.2082	0.1826	0.1845	0.1857	–	0.6758	0.6988	0.7015	0.7463	0.6680	0.7625	0.7510
GL	0.3744	0.4075	0.3477	0.4349	0.3898	0.3763	0.3899	0.3919	–	0.7782	0.7180	0.6492	0.7853	0.6957	0.7037
GZ	0.3618	0.3854	0.3674	0.3821	0.3539	0.3643	0.3769	0.3584	0.2508	–	0.8073	0.6588	0.7349	0.7222	0.7144
DL	0.3729	0.3830	0.3503	0.3583	0.3424	0.3597	0.3577	0.3546	0.3313	0.2141	–	0.6553	0.7561	0.7484	0.7243
NJC	0.2530	0.3422	0.2943	0.2771	0.2598	0.2787	0.2889	0.2927	0.4320	0.4173	0.4226	–	0.6271	0.7537	0.7227
MAS	0.3752	0.3856	0.3612	0.4323	0.4138	0.3829	0.4135	0.4034	0.2416	0.3080	0.2796	0.4667	–	0.6965	0.6889
LY	0.2282	0.2838	0.2506	0.2610	0.2193	0.2047	0.2230	0.2712	0.3628	0.3255	0.2898	0.2828	0.3617	–	0.7705
LA	0.2770	0.3083	0.2928	0.3053	0.2596	0.2372	0.1969	0.2863	0.3515	0.3363	0.3225	0.3248	0.3727	0.2607	–
(b) SRAP															
SY	–	0.8277	0.8814	0.8290	0.9050	0.8457	0.8391	0.8309	0.6926	0.6986	0.7086	0.8176	0.6802	0.7846	0.7738
HS	0.1891	–	0.8359	0.7650	0.8271	0.8027	0.7806	0.7661	0.6732	0.6833	0.6859	0.7517	0.6993	0.7497	0.7225
JH	0.1263	0.1793	–	0.8454	0.8670	0.8619	0.8488	0.8417	0.7152	0.7226	0.7350	0.8102	0.6928	0.7954	0.7601
TXW	0.1876	0.2679	0.1679	–	0.8504	0.8430	0.8142	0.8186	0.7174	0.7109	0.7147	0.7704	0.6878	0.8989	0.7580
DF	0.0999	0.1898	0.1427	0.1620	–	0.8817	0.8330	0.8358	0.7079	0.7117	0.7217	0.8031	0.6986	0.8001	0.7656
TXC	0.1676	0.2198	0.1486	0.1708	0.1260	–	0.8342	0.8420	0.7054	0.7173	0.7272	0.8178	0.6857	0.7859	0.7672
JR	0.1754	0.2477	0.1640	0.2055	0.1827	0.1812	–	0.9298	0.7213	0.7390	0.7472	0.8029	0.7052	0.7782	0.8572
SQ	0.1852	0.2665	0.1723	0.2002	0.1794	0.1719	0.0728	–	0.7583	0.7518	0.7596	0.8126	0.7095	0.7600	0.8262
GL	0.3673	0.3957	0.3352	0.3321	0.3454	0.3490	0.3267	0.2766	–	0.8341	0.8324	0.6840	0.7785	0.7063	0.6971
GZ	0.3587	0.3808	0.3249	0.3412	0.3401	0.3322	0.3025	0.2853	0.1814	–	0.8996	0.6852	0.8372	0.7010	0.7029
DL	0.3445	0.3770	0.3079	0.3358	0.3262	0.3186	0.2914	0.2749	0.1834	0.1058	–	0.7238	0.8566	0.7003	0.6951
NJC	0.2014	0.2854	0.2105	0.2609	0.2193	0.2012	0.2196	0.2075	0.3798	0.3780	0.3232	–	0.6817	0.7179	0.7130
MAS	0.3854	0.3576	0.3670	0.3742	0.3587	0.3774	0.3493	0.3432	0.2504	0.1777	0.1548	0.3832	–	0.6842	0.6749
LY	0.2426	0.2881	0.2288	0.1065	0.2230	0.2409	0.2508	0.2744	0.3477	0.3553	0.3563	0.3315	0.3795	–	0.7476
LA	0.2564	0.3251	0.2743	0.2771	0.2671	0.2651	0.1540	0.1909	0.3608	0.3525	0.3637	0.3383	0.3932	0.2909	–

(a) Based on the ISSR analysis; (b) Based on the SRAP analysis. Above diagonal: Nei's unbiased measures of genetic identity; below diagonal: genetic distance.

3.4. Combined data analysis

The relationships between the 15 populations revealed that all the populations were grouped into 6 main clusters by the cluster analysis (Fig. 2). The first cluster includes "SY", "DF", "JS", "TXC", "JR" and "SQ"; the second includes "TXW" and "LA"; the third includes only "HS"; the fourth includes only "LA"; the fifth cluster includes only "NJC"; and the sixth cluster includes "GZ", "DL", "GL" and "MAS". For this dendrogram (Fig. 2c), the cophenetic correlation was estimated to be $r = 0.90404$, which corresponds to a very good fit. The results of the PCA analysis were comparable to those obtained from the cluster analysis. The three most informative PC components explained 47.2% of the total variation.

The ISSR and SRAP markers exhibit comparable Ibav, EMR and MI values of 0.3490 and 0.3687, 11.6 and 14.4, 4.04 and 5.3, respectively. However, the calculated MI was higher for the SRAP than for the ISSR, which highlights the unique advantage of the high multiplex ratio provided by the SRAP technique.

Both two markers proved to be highly effective in discriminating the fifteen populations used in this study. Some differences in ISSR markers were apparent and contrasted with the SRAP. To obtain a more convincing comparison, the Mantel matrix correspondence test was used in matrices of cophenetic values that were generated from the ISSR and SRAP dendrograms. The correlation coefficients were statistically significant for both two marker systems. The correlations between the ISSR and SRAP dendrograms were 0.8564 ($P < 0.01$).

4. Discussion

Application evaluations using molecular markers are basic for analyses of genetic diversity (Milbourne et al., 1997). From Jaccard's genetic similarity coefficient and the UPGMA clustering displays, it is known that selected populations are obviously divided into different groups. In this study, two markers could distinguish *W. pigra* and *H. nipponica*. The ISSR and SRAP markers showed relatively high polymorphisms among the 15 populations in this study, which indicates that this analysis was effective based on the clustering of the two markers, and the similarity of the regional differences were highly consistent. Because the technology and materials used in studying these two markers are different, the conclusions that are reached for each marker might not be the same. Researchers have examined the existence of correlations between different molecular marker techniques in various species. Yilmaz et al. (2012) reported similar genetic relationships among Turkish apricot germplasms using three marker systems. Archak et al. (2003) believed that mating methods can significantly affect the degree

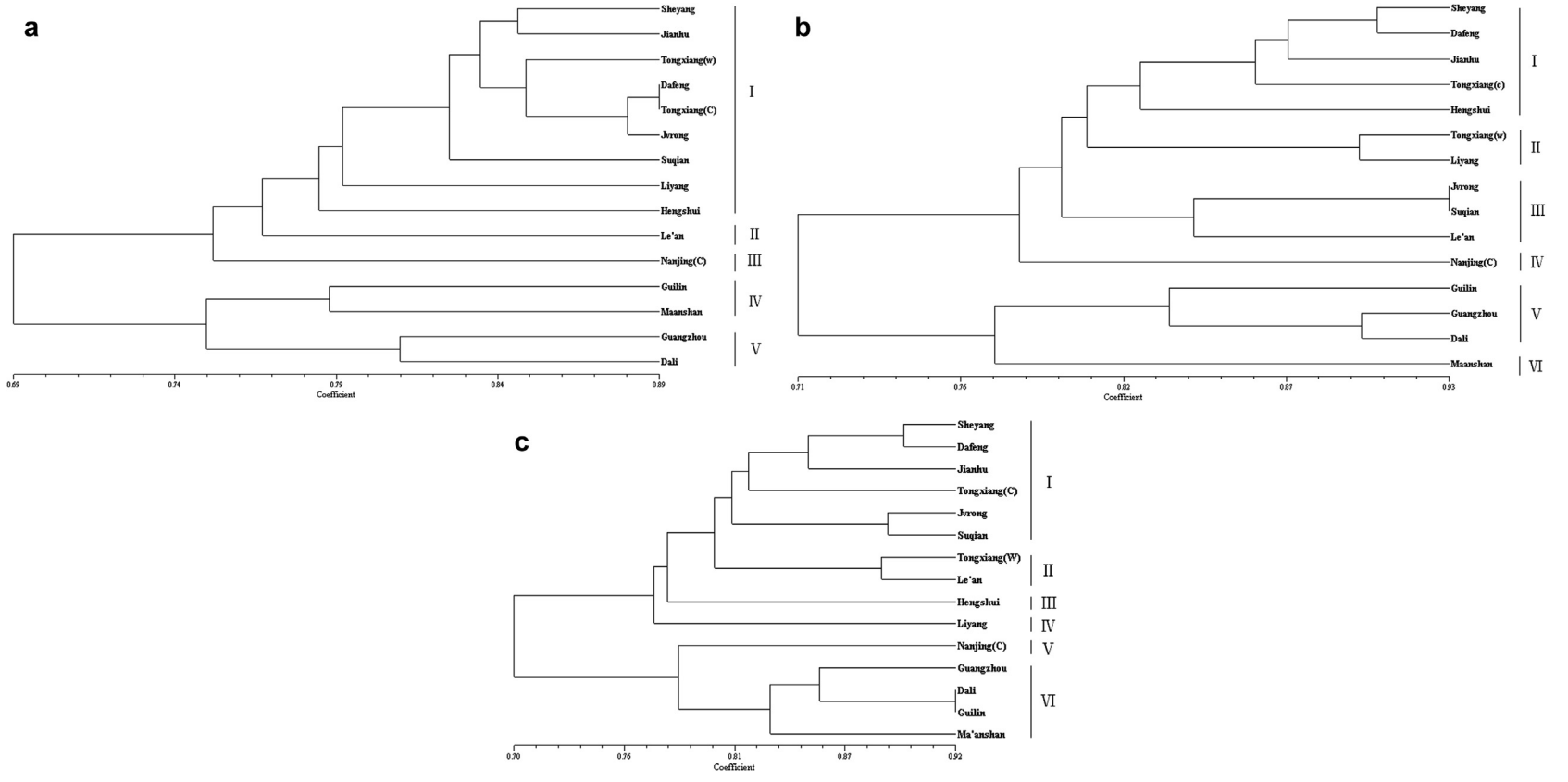

Fig. 2. (a) UPGMA dendrogram determined from the ISSR using the unweighted pair-group method with arithmetic average cluster analysis; (b) UPGMA dendrogram determined from the SRAP using the unweighted pair-group method with arithmetic average cluster analysis; (c) UPGMA dendrogram determined from the ISSR and SRAP combined date using the unweighted pair-group method with arithmetic average cluster analysis.

of correlation between markers. Correlation coefficients of a similar magnitude were also found among the AFLP, ISSR and SSR markers by Paris et al. (2003) in *Cucurbita pepo* (Cucurbitaceae) and among the AFLP, RAPD and RFLP markers that were compared by Garcia-Mas et al. (2000) in *Cucumis melo*. In the present study, ISSRs and SRAPs demonstrated a notable correlation and displayed different polymorphism capacities, which were detected through analyses of marker efficiencies. In the present study, we obtained a good degree of correlation for the two molecular markers. According to interpretations ($r \geq 0.9$ Very good fit, $0.8 \leq r < 0.9$ Good fit, $0.7 \leq r < 0.8$ Poor fit, $r < 0.7$ Very poor fit) (Mantel, 1967), ISSR and SRAP displayed a good fit, indicating that the molecular data obtained using the ISSR and SRAP markers were accurate and reliable for the study of genetic diversity.

Among the 15 populations, SRAP had a higher MI value than ISSR, which indicates that the former had a higher marker efficiency than the latter. From comparing the results of the MI component parameters, SRAP had more polymorphic bands, indicating that its EMR value was high, which might be the main reason that the MI value for SRAP was higher than the values for ISSR. A relatively high MI value was obtained for SRAP. The EMR and Ibav values were not the main factors that determined the marker efficiency, indicating that the average band number was responsible for the alterations in the marker efficiency. Ibav values are a revised method to calculate the diversity index (Fernandez et al., 2002) and represent a direct manifestation of the amplification degree of a polymorphic band. Ibav values indicate that the SRAP markers are highly polymorphic among species with a close relationship (MacRitchie and Sun, 2004; Sun et al., 2002), which is applicable to the analysis of interspecies or species with a close relationship. ISSRs have a high capacity to reveal polymorphisms and offer significant potential for determining intra- and inter-genomic diversities compared to other arbitrary primers, which is in contrast with the results of this study.

The evolutionary potential of a species and its ability to withstand adversity depend on the degree of intra-specific genetic variation. Species with a higher genetic diversity will have a stronger environmental adaptability and wider natural distribution. Genetic diversity is essential for the long-term survival of a species and its adaptability to the environment. Thus, before making any protection and management strategies for an endangered species, it was very important to be aware of the species' genetic diversity among and within species (Hamrick and Godt, 1996). Accordingly, it can be assumed that the genetic diversity of the 15 *Hirudo* populations investigated, as determined by detecting the two molecular markers, was relatively not very high, which also indicates that the level of genetic structure among the populations is relatively not very high, according to Nei's genetic diversity analysis.

The genetic structures of *W. pigra* and *H. nipponica* partly result from the interaction of gene flow and genetic drift (Hutchison and Templeton, 1999). Generally, gene flow ($Nm = 1$) can prevent the genetic differentiation of a population due to genetic drift (Slatkin, 1987). In this study, the gene flow (Nm) of the population was 0.3727 by ISSR and 0.5641 by SRAP. The Nms for both two markers were less than 1, indicating that the gene flow among these populations was very small and would not be able to resist the population differentiation caused by interspecies' genetic drift (Slatkin, 1985). Hence, genetic drift might be the major factor that causes *W. pigra* and *H. nipponica* populations to experience significant genetic differentiation. A rich level of genetic diversity for a population is typically inferred to be related to both its genetic drift and its breeding methods. The low gene flow rates for *W. pigra* and *H. nipponica* might be predominantly caused by the breeding methods used. *Hirudo* are hermaphrodites and reproduce through an allogeneic mating scheme type of sexual reproduction; however, their hermaphroditic behaviour in itself still indicates that *Hirudo* are not completely evolved. mtDNA is inherited maternally, and studies based on its control sequences cannot reflect the flow of individual male genes. In addition to breeding methods and genetic drift, the observed genetic differentiation might also be caused by such factors as geographic isolation, the ecological environment, population and history.

In conclusion, the present study highlights the usage of ISSR and SRAP techniques for studying genetic diversity across the DNA level in *W. pigra* and *H. nipponica*. To the best of such knowledge, no such studies have yet been reported on comparisons of the discriminating capacity, efficiency and ability of the ISSR and SRAP marker systems in *W. pigra* and *H. nipponica*. These results will be utilised as new information for future studies on the phylogenetic relationships, cultivar identification and germplasm organisation of genus *Hirudo*.

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References

Archak, S., Gaikwad, A.B., Gautam, D., Rao, E.V., Swamy, K.R., Karihaloo, J.L., 2003. Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. Genome 46, 362–369.

Bassam, B.J., Caetano-Anollés, G., Gresshoff, P.M., 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196, 80–83.

Budak, H., Shearman, R.C., Parmaksiz, I., Gaussoin, R.E., Riordan, T.P., Dweikat, I., 2004. Molecular characterization of buffalograss germplasm using sequence related amplified polymorphism markers. Theor. Appl. Genet. 108, 328–334.

Chinese Pharmacopoeia Editorial Committee, 2010. The Pharmacopoeia of the People's Republic of China: I. Chemical Industry Press, Beijing, p. 77.

Dice, L.R., 1945. Measures of the amount of ecological association between species. Ecology 26, 297–302.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: applications to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Fernandez, M.E., Figueiras, A.M., Benito, C., 2002. The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. Theor. Appl. Genet. 104, 845–851.
Garcia-Mas, J., Oliver, M., Gomez-Paniagua, H., De-Vicente, M.C., 2000. Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. Theor. Appl. Genet. 101, 860–864.
Guo, Q.S., Liu, F., Shi, H.Z., 2006. Residues analysis of pesticides and heavymetals in *Whitmania pigra* and its breeding base. Chin. J. Chin. Mat. Med. 31, 1763–1765.
Hamrick, J.L., Godt, M.J.W., 1996. Effects of life history traits on genetic diversity in plant species. Phil. Trans. R. Soc. Lond. B 351, 1291–1298.
Hutchison, D.W., Templeton, A.R., 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53, 1898–1914.
Jin, W.J., Zhang, R.J., 2002. Pressure sensation by an anterior pagoda neuron population distributed in multiple ganglia in the leech, *Whitmania pigra*. J. Comp. Physiol. A 188, 165–171.
Kasparek, M., Demirsoy, A., Akbulut, A., Akbulut, N., Çali-kan, M., Durmu, Y., 2000. Distribution and status of the medicinal leech (*Hirudo medicinalis* L.) in Turkey. Hydrobiologia 441, 37–44.
Li, G., Quiros, C.F., 2001. Sequence-related amplified polymorphism (SRAP), A new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet. 103, 455–461.
Liu, F., Guo, Q.S., Shi, H.Z., Wang, T., Lu, S.S., 2010. Comparation study on quality of different population of wild and breeding *Whitmania pigra*. Chin. J. Chin. Mat. Med. 35, 1280–1283.
Liu, F., Shi, H.Z., Guo, Q.S., Wang, T., Lu, S.S., 2011. Analysis of genetic differentiation and phylogenetic relationship between *Whitmania pigra* and *Hirudo nipponica* based on ITS sequence. Chin. J. Chin. Mat. Med. 36, 414–419.
MacRitchie, D., Sun, G.L., 2004. Evaluating the potential of barley and wheat microsatellite markers or genetic analysis of Elymus trachycaulus complex species. Theor. Appl. Genet. 108, 720–724.
Mantel, N., 1967. The detection of disease clustering and generalized regression approach. Cancer Res. 27, 209–220.
McGregor, C.E., Lambert, C.A., Greyliing, M.M., Louw, J.H., Warnich, L., 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 113, 135–144.
Milbourne, D., Rhonda, M., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powell, W., Waugh, R., 1997. Comparison of PCR-based marker systems for the analysis of genetic relationships in cultivared potato. Mol. Breeding 3, 127–136.
Nei, M., 1972. Genetic distance between populations. Am. Nat. 106, 283–292.
Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. P. Natl. Acad. Sci. USA 76, 5269–5273.
Nikonov, G.I., Titova, E.A., Seleznev, K.G., 1999. Biological activity of pharmacological properties of the anticoagulant complex (hirudin, plasma kallikrein inhibitor, prostaglandin) from the leech Hirudo medicinalis. Biull. Eksp. Biol. Med. 128, 673–676.
Ozono, R., 2006. New biotechnological methods to reduce oxidative stress in the cardiovascular system: focusing on the Bach1/heme oxygenase-1 pathway. Curr. Pharm. Biotechnol. 7, 87–93.
Paris, H.S., Yonash, N., Portnoy, V., Mozes-daube, N., Tzuri, G., Katzir, N., 2003. Assessment of genetic relationships in *Cucurbita pepo* (Cucurbitaceae) using DNA markers. Theor. Appl. Genet. 106, 971–978.
Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A., 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol. Breeding 2, 225–238.
Prevost, A., Wilkinson, M.J., 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. 98, 107–112.
Rohlf, F.J., 2004. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System Version 2.1 User Guide. Exeter Software. Applied Biostatistics Inc, New York.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning: a Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York, pp. 21–66.
Shao, Q.S., Guo, Q.S., Deng, Y.M., Guo, H.P., 2010. A comparative analysis of genetic diversity in medicinal Chrysanthemum morifolium based on morphology, ISSR and SRAP markers. Biochem. Syst. Ecol 38, 1160–1169.
Shen, D.W., Lu, Q.W., Wang, L., 2002. Ultrastructure of electrical synapses between nociceptive and anterior pagoda neurons in the CNS of the leech (*Whitmania pigra*). Invert. Neurosci. 4, 193–198.
Shi, H.Z., Liu, F., Guo, Q.S., 2006. Studies impact of temperature and weight in *Whitmania pigra* Bred. Chin. J. Chin. Mat. Med. 31, 2030–2032.
Shi, H.Z., Guo, Q.S., Liu, F., Li, H.J., Zhao, S.S., 2007. Studies on internal quality about different processing of wild and the cultivated *Whitmania pigra*. Chin. J. Chin. Mat. Med. 32, 2657–2659.
Slatkin, M., 1985. Gene flow in natural populations. Annu. Rev. Ecol. Syst. 16, 393–430.
Slatkin, M., 1987. Gene flow and the geographic structure of natural populations. Science 236, 787–792.
Sun, G.L., Salomon, B., Von-Bothmer, R., 2002. Microsatellite polymorphism and genetic differentiation in three Norwegian populations of *Elymus alaskanus* (Poaceae). Plant. Syst. Evo. 234, 101–110.
Yeh, F.C., Yang, R.C., Boyle, T.B.J., Ye, Z.H., Mao, J.X., 1999. PopGene Version 3.2: The User-friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Center, University of Albert, Edmonton.
Yilmaz, K.U., Paydas-Kargi, S., Dogan, Y., Kafkas, S., 2012. Genetic diversity analysis based on ISSR, RAPD and SSR among Turkish Apricot Germplasms in Iran Caucasian eco-geographical group. Sci. Hortic. 138, 138–143.
Zhang, B., Lin, Q., Lin, J.D., Chu, X.L., Lu, J.Y., 2008. Effects of broodstock density and diet on reproduction and juvenile culture of the leech, *Hirudinaria manillensis* Lesson, 1842. Aquaculture 276, 198–204.