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Isolation and characterization of microsatellite loci in the Korean crayfish, *Cambaroides similis* and application to natural population analysis

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The Korean freshwater crayfish, *Cambaroides similis*, has recently suffered from range reduction and habitat degradation caused by environmental changes and water pollution. For the conservation and restoration of this species, it is necessary to understand the current population structures of Korean *C. similis* using estimation of their genetic variation. In this study, eight microsatellite loci were developed and characterized from 49 individuals collected from four locations: one population from Mt. Bukhan (BH) and three populations from Mt. Gwanak (GA) in Seoul, Korea. As a result, the number of alleles per locus ranged from 2 to 12. The observed heterozygosities and expected heterozygosities ranged from 0.000 to 0.833 and from 0.125 to 0.943, respectively, and the former values were significantly lower than the latter ones expected under the Hardy-Weinberg equilibrium. No significant linkage disequilibrium was revealed between any of the locus pairs after Bonferroni correction. From the pairwise *Fst* results over all samples, higher differentiation between GA-BH population pairs (mean 0.1789) was observed than between GA population pairs (mean 0.0454). This was also supported by Mantel's test showing that the genetic distances of these crayfish populations were significantly correlated with geographic distances. This result may show the regional differentiation caused by restricted gene flow between northern (BH) and southern (GA) populations within Seoul. These microsatellite markers have the potential for use in analyses of the genetic diversity and population structure of *C. similis* species, with implications for its conservation and management plans.

Keywords: microsatellite loci; freshwater crayfish; *Cambaroides similis*; Korea; population genetics

Introduction

Crayfishes (Decapoda, Astacidea) are the largest invertebrates in most freshwater habitats and play an important role in the food webs and ecosystems as dominant consumers of benthic invertebrates, detritus, algae and water plants (Lodge et al. 2000). The crayfish fishery in many countries constitutes a substantial social, cultural, recreational and economic value (Edsman et al. 2002; Gherardi et al. 2003). Recently, however, many crayfish species or local populations have declined and become endangered around the world, due to factors such as the destructive human impact on the environment (including acidification and pollution), invasion of alien crayfish species, introduction of fatal crayfish plague (*Aphanomyces astaci*), and water shortages or drought (Lodge et al. 2000; Taylor 2002; Edgerton et al. 2004). For more effective conservation plans, many studies of a genetic approach using molecular markers such as microsatellites and mitochondrial DNA sequences have been conducted for many crayfish groups including threatened or vulnerable species in the world (Gouin et al. 2000, 2006; Zaccara et al. 2005; Crandall et al. 2009).

The East-Asian freshwater crayfish genus *Cambaroides* (Faxon, 1885) comprises four species and has a wide distribution in the Far Eastern Asia, including Korea, Japan and eastern parts of China and Russia. Among these species, *Cambaroides similis* (Koelbel, 1892) is the only species of crayfish native to Korea and adjacent northern areas. Most of the habitats of this species were restricted to small brooks or puddles that form on the upper edge of mountain streams (Kawai and Min 2005). Although this species is distributed widely across the middle and southern parts of the Korean Peninsula, its populations have been continuously influenced by environmental changes and water pollution (Ahn et al. 2006; Kim et al. 2006). Moreover, owing to the water shortage or dryness in the habitats caused by frequent droughts during the winter and early spring and by over-consumption of subterranean waters in recent years, the Korean crayfish populations may have decreased drastically or even disappeared.

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For the conservation and restoration of this species, it is necessary to understand the current population structures of Korean *C. similis* using estimation of their genetic variation (Kim et al. 2006). Since little is known about the genetic properties of Korean crayfish populations, conservation measures and efforts are urgently needed to preserve this endangered species. In this study, we describe first the development and characterization of eight polymorphic microsatellite loci from the Korean crayfish *C. similis*, which are valuable to assess genetic diversity and structure at the population or regional scale. We applied these markers to natural population analysis of Korean crayfish populations.

Materials and methods

Specimens of the Korean freshwater crayfish, *C. similis* (n = 49) were collected from three locations on Mt. Gwanak (sub-population GA1, n = 11; GA2, n = 8; GA3, n = 12) and one on Mt. Bukhan (BH, n = 18) in Seoul, South Korea (Figure 1). Total genomic DNA was extracted from the muscle tissue of ethanol-preserved specimens using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA). In particular, genomic DNA from an individual in the Gwanak (GA2) population was used in the microsatellite maker development. The extracted DNA was stored at −20°C until use.

Microsatellites were developed using the enrichment protocol described by Hammond et al. (1998) with minor modifications. Briefly, we digested genomic DNA with restriction enzyme *Mbo*I (Promega, Madison, WI). Next, the resulting fragments were ligated to SAUL linkers (Hammond et al. 1998) using T4 DNA ligase (Promega). The ligated fragments were then enriched for microsatellites using a cocktail comprised of seven biotinylated probes ($(AG)_{15}$, $(AC)_{15}$, $(ACG)_{10}$, $(CAA)_{10}$, $(GAA)_{10}$, $(ACAT)_7$, $(AGAT)_7$) bound to streptavidin magnetic beads (Streptavidin MagneSphere® Paramagnetic Particles, Promega). This enrichment process was performed twice. The enriched fragments were then amplified and cloned using the pGEM®-T Easy Vector System (Promega). The size of the insert in 259 clones was evaluated by polymerase chain reaction (PCR) using the linker primer, and clones showing insert sizes ranging from 200 bp to 700 bp were selected for sequencing. Fifty-nine clones were subjected to double-stranded DNA sequencing using BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3730xl sequencer (Applied Biosystems).

A total of 25 PCR primer pairs were designed using Primer 3 software (Rozen and Skaletsky 2000). Of the developed primer pairs, eight microsatellite loci were characterized with 49 individuals that had been collected from four wild populations in South Korea. Two different labeling methods were applied to these eight primer pairs. Specifically, each forward primer specific for five loci (MSCS-1, MSCS-2, MSCS-3, MSCS-4, and MSCS-5) was labeled individually with the fluorescent dye PET, 6FAM or VIC. In addition, the M13 (-21) (5′-TGTAAAACGACGGCCAGT-3′) sequence tag method was used to label the primers specific for MSCS-11, MSCS-13 and MSCS-17 (Schuelke 2000) (Table 1).

Each microsatellite locus was tested by PCR amplification which was conducted using a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems). Each reaction mixture contained 200 μm dNTPs (GeneCraft, Köln, Germany), 1 × PCR buffer with 1.5 mm $MgCl_2$, 1 U of *Taq* DNA polymerase

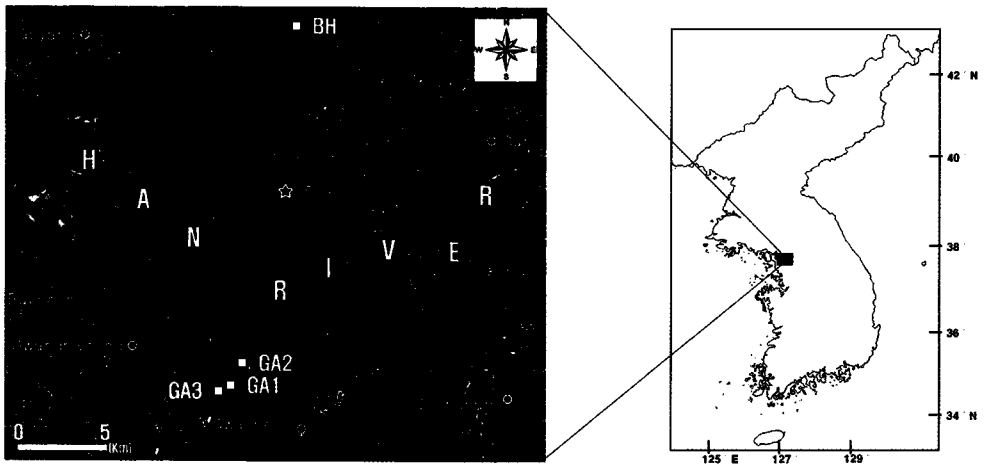

Figure 1. Sampling locations of the four Korean populations of *Cambaraides similis* used in this study (BM in Mt. Bukhen; GA1, GA2, GA3 in Mt. Gwanat).

Table 1. Characteristics of eight microsatellites isolated from *Cambaroides similis*. Shown are the locus name, repeat motif, primer sequence, GenBank accession number, and specific annealing temperature (°C).

Locus	Repeat motif	Primer sequence (5′-3′) (F = forward; R = reverse)	GenBank Accession no.	Annealing *T*m (°C)
MSCS-1	$(CT)_{43}$	F: ^{PET}CTGAACTCTGTATTGGAGGGTAG R: CGGTAGTTTTAACAGGTCTGGG	FJ230081	55
MSCS-2	$(AC)_{14}T(AC)_2$	F: ^{PET}GAACACTCTACCCTGCCAG R: TACAGTTTCTGTAGGAAAGTAAAC	FJ230082	58
MSCS-3	$(AGTG)_{28}$	F: ^{6FAM}CTGTAAGAGCTTCTACAATGG R: CAAAAGGCGTTCACTCTTGG	FJ230083	58
MSCS-4	$(CT)_{36}$	F: ^{VIC}ACAACAAGGGGCCAAGCAC R: ACCTGGGATGCGATGCCTGCC	FJ230084	60
MSCS-5	$(AC)_{28}$	F: ^{6FAM}ACACCGGCGACTGTAGGCAG R: TTGGTGTAGCGCAGAGTGACG	FJ230085	60
MSCS-11	$(AG)_{36}$	F: ^{6FAM}†CCATTAAACCCCCGCACCTA R: ATCCCAAGCTTCATTGGCTC	FJ230088	55
MSCS-13	$(TC)_{36}$	F: ^{6FAM}†GTAGATGGGGGCTGGCAACAG R: AACTTGACCGTTGAAGGTCTCACG	GQ168720	56
MSCS-17	$(CT)_{50}$	F: ^{PET}†TTAGCTGTACTTGTGTAG R: ATTCCTAATGAACCCTAG	GQ168723	55

† Primers tagged with M13(-21) (5′-TGTAAAACGACGGCCAGT-3′) tail.

(TaKaRa, Otsu, Japan), 10 ng of DNA and an appropriate concentration of primers in a total volume of 30 μL. The concentration of the primers differed depending on the primer pair that was used. When the individually labeled forward primers were used, 0.3 μM of each forward and reverse primer was added to the PCR reaction. However, when the M13(-21) tagged primers were used, the mixture contained 0.08 μM forward M13(-21) tagged primer, 0.3 μM reverse primer, and 0.3 μM M13(-21) fluorescently labeled primer. The PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 30 s at 95°C, 45 s at 55–60°C and 1 min at 72°C, with a final extension at 72°C for 10 min. Fluorescently labeled PCR products were electrophoresed concurrently with the GeneScan™-500LIZ™ Size Standard (Applied Biosystems) on an ABI 3730xl sequencer (Applied Biosystems). The size was then determined using GENEMAPPER version 3.7 (Applied Biosystems).

The MICRO-CHECKER version 2.2.3 software (Van Oosterhout et al. 2004) was used to identify possible genotyping errors (i.e. null alleles, stuttering and large allele dropout) which can cause deviations from Hardy-Weinberg proportions, in particular heterozygote deficiency. The number of alleles, the allelic size range, the diversity statistics (observed and expected heterozygosities), the Hardy-Weinberg equilibrium for each locus, tests for genotypic linkage disequilibrium, the inbreeding coefficient (*F*is) (Weir and Cockerham 1984) per locus and sample, along with assessments of multi-locus *F*st values were carried out using GENEPOP version 4.0 software (Rousset 2008). Corrections of the significance level for multiple tests were adjusted using the Bonferroni procedure (Rice 1989). The correlation of genetic distances over geographical distances for all pairs of populations was tested using a Mantel's test which implemented in Isolation by Distance Web Service (IBDWS) version 3.15 (Jensen et al. 2005).

Results and discussion

Development and characterization of microsatellite loci

From a total of 259 colonies screened using a PCR-based technique, we summarized the characteristics of eight newly developed microsatellite loci which were found to be polymorphic in Korean *C. similis* populations: repeat motifs, primer sequences, specific annealing temperatures, and a fluorescent label for each locus are shown in Table 1. Among these microsatellite loci, only locus MSCS-3 was a tetranucleotide repeat, while the others were dinucleotide repeats.

Forty-nine individuals collected from three Mt. Gwanak (GA1, GA2, and GA3) and one Mt. Bukhan (BH) populations were screened to verify the genetic variation of these eight microsatellite loci. All individuals were successfully amplified. The overall polymorphic loci, the number of alleles per locus, ranged from 2 to 12 (Table 2). A total of 106 different alleles were observed over all eight microsatellite loci composed of GA1 (39 alleles), GA2 (34 alleles), GA3 (47 alleles) and 65 alleles in the BH population.

The Micro-Checker analysis showed that loci MSCS-3, 4, 17 in GA1, loci MSCS-1, 17 in GA2, loci

Table 2. Summary of variation across eight microsatellite loci in Korean crayfish (*C. similis*) from four natural populations: number of alleles (*A*), allele size range (*R*), expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (*Fis*).

		Microsatellite loci								
Population (*n*)		MSCS-1	MSCS-2	MSCS-3	MSCS-4	MSCS-5	MSCS-11	MSCS-13	MSCS-17	Average across loci
	A	6	3	9	6	5	2	3	5	4.9
GA1 ($n=11$)	*R*	135–181	92–96	154–202	208–236	220–244	306–308[a]	115–193[a]	278–292[a]	–
	H_E	0.873	0.591	0.900	0.827	0.636	0.182	0.255	0.809	0.634
	Ho	0.636*	0.455	0.545*	0.455**	0.455	0.000*	0.273	0.091**	0.364
	Fis	0.271	0.231	0.394	0.450	0.286	1.000	–0.071	0.887	0.431
	A	5	2	7	4	3	2	5	6	4.3
GA2 ($n=8$)	*R*	159–173	94–96	154–206	202–218	220–238	306–308[a]	115–199[a]	272–290[a]	–
	H_E	0.821	0.518	0.911	0.696	0.357	0.125	0.759	0.911	0.637
	Ho	0.125**	0.625	0.625**	0.375	0.125	0.125	0.500	0.125**	0.328
	Fis	0.848	−0.207	0.314	0.461	0.650	0.000	0.341	0.863	0.409
	A	8	3	11	7	6	2	3	7	5.9
GA3 ($n=12$)	*R*	135–181	92–96	146–210	204–222	220–240	308–310[a]	115–193[a]	278–294[a]	–
	H_E	0.894	0.300	0.943	0.841	0.686	0.303	0.235	0.890	0.637
	Ho	0.500**	0.333	0.417**	0.417**	0.583	0.000**	0.250	0.333**	0.354
	Fis	0.441	−0.114	0.558	0.504	0.149	1.000	−0.064	0.625	0.387
	A	12	3	9	10	7	3	12	9	8.1
BH ($n=18$)	*R*	165–237	92–96	150–210	196–222	206–254	306–310[a]	145–295[a]	230–278[a]	–
	H_E	0.872	0.542	0.781	0.889	0.825	0.212	0.900	0.846	0.733
	Ho	0.611**	0.500	0.388**	0.389**	0.667**	0.111	0.278**	0.833	0.472
	Fis	0.299	0.078	0.502	0.562	0.192	0.477	0.691	0.015	0.352

* Significant deviation ($P < 0.05$) and ** ($P < 0.01$) from the Hardy-Weinberg equilibrium. [a] Size excluding a 18 bp M13(-21) tail.

MSCS-1, 3, 4, 11, 17 in GA3 and loci MSCS-1, 3, 4, 13 in the BH population were prone to null alleles. There was no evidence of large allele dropout in any of the loci, but only one locus in the GA1 (MSCS-17), GA3 (MSCS-11) and BH populations (MSCS-4) appeared to be influenced by scoring error due to stuttering.

Observed heterozygosities (H_O) ranged from 0.000 to 0.833, whereas expected heterozygosities (H_E) varied from 0.125 to 0.943 over all four populations (Table 2). The observed heterozygosities in four populations were significantly lower than the heterozygosity value expected under the Hardy-Weinberg equilibrium (HWE). Previous genetic studies using allozyme variations suggested that freshwater crayfish species in general comprise an unusually low level of heterozygosity compared to other invertebrates (Crandall 1997).

The inbreeding coefficients (*Fis*) varied among loci from –0.071 to 1.000 in GA1, from –0.207 to 0.863 in GA2, from –0.114 to 1.000 in GA3, and from 0.015 to 0.691 in the BH population (Table 2). The average *Fis* including all markers was 0.431 in GA1, 0.409 in GA2, 0.387 in GA3 and 0.352 in the BH population. In addition, no significant linkage disequilibrium was revealed between any of the locus pairs following Bonferroni correction ($P < 0.00625$).

Heterozygosity deficit in local populations

In this study, many loci showed significant deficiency of heterozygosity, meaning deviations from the HWE, over all four populations. Significant deviations from the HWE were observed at three loci (MSCS-2, 5, 13) in the GA1 population, at four loci (MSCS-2, 4, 5, 13) in GA2, at three loci (MSCS-2, 5, 13) in GA3 and at three loci (MSCS-2, 11, 17) in the BH population. We supposed two possible causes for the high deficiency of heterozygosity in the studied populations, with the possible presence of null alleles. The first reason may be inbreeding due to the low capacity for natural dispersal and the interruption of the gene flow by habitat fragmentation and isolation. This can happen easily because most habitats of Korean crayfishes are restricted to small and narrow mountain ravines which are vulnerable to environmental changes or destruction.

As an individual of *C. similis* can produce more than 50 eggs in each spawning (Kim 1977), it is highly possible that mating between relatives occurred. Therefore, it appears that the deficiency of heterozygosity within each population observed in this study was caused by non-random mating in an isolated and limited habitat range (Li et al. 2004; Yue et al. 2010). The second reason is the fact that the structures of these Korean crayfish populations are highly unstable, with the effects of habitat alteration and reduction. Thus the possibility of a Wahlund effect caused by the

Table 3. Pairwise *Fst* values (below diagonal, bold) and geographic distances (above diagonal, km) between Korean four *Cambaroides similis* populations based on eight microsatellite loci.

Population	GA1	GA2	GA3	BH
GA1		1.67	0.30	22.67
GA2	**0.0731**		1.77	21.35
GA3	**–0.0039**	**0.0671**		22.63
BH	**0.1813**	**0.1523**	**0.2030**	

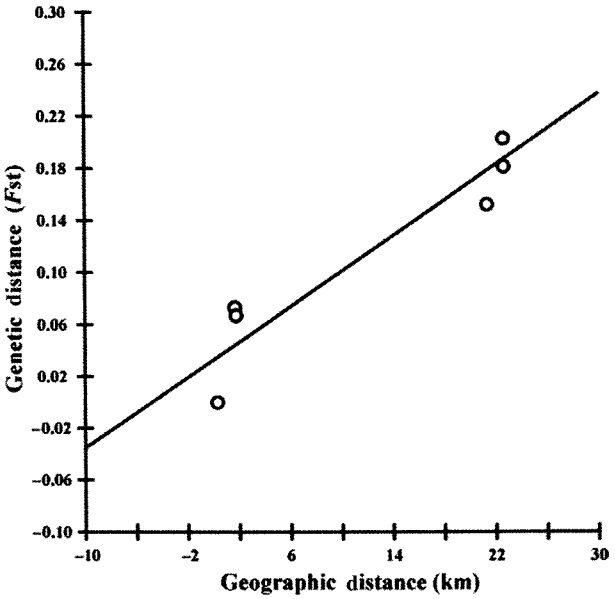

Figure 2. Correlation between genetic distance (Fst value) and geographic (kilometers) distance ($r = 0.943$) performed by Mantel's test.

opportunistic individual immigration from adjacent crayfish populations cannot be overlooked. These habitat patterns were similar to those of a 'meta-population', which is confined to a network of geographically separated habitat patches that may be recolonized through dispersal of individuals from other patches (Buckley and Pollett 2010). Considering a meta-population, a new approach to Korean crayfish populations may be necessary in the future. Although the sample size of the *C. similis* populations used in this study is too small to yield reliable results, these microsatellite primers must have the potential to capitalize on the usefulness of these loci in genetic variation and population structure studies of *C. similis*.

Genetic differentiation between Gwanak (GA) and Bukhan (BH) populations

All of the sampling locations in the present study belong to the Han River water system in Korea. However, they are largely separated into two drainage systems: a southern system (Mt. Gwanak, GA) and a northern system (Mt. Bukhan, BH). The distance between the three sub-populations (GA1, GA2, and GA3) on Mt. Gwanak is only 2 km in each case, while the distance between GA and BH populations is about 22 km. However, at present these populations are disconnected by habitat fragmentation and disturbances due to land use in urban (downtown) regions in which crayfishes cannot live naturally.

We estimated the genetic differentiation (average *F*st value) between these populations. From the results of the average *F*st over all samples, higher differentiation between GA-BH population pairs (mean 0.1789) was observed compared to that between GA population pairs (mean 0.0454) (Table 3). In addition, the result of isolation-by-distance indicated a significant correlation between genetic distance and geographic distance ($r = 0.943$, $P = 0.085$), as well as significant isolation by distance based on Mantel's test (from 30,000 randomizations) (Figure 2). Genetic differences that are caused by geographic isolation are important and the geographic distances are roughly related to the genetic distance (Versteegen and Lawler 1996). Our results may show the regional genetic differentiation caused by restricted gene flow among Korean crayfish populations.

Conclusion

The eight microsatellite markers described in the present study will be useful for studying the genetic diversity and differentiation within and among additional extensive *C. similis* populations. Moreover, these markers are expected to be useful for further studies of the population genetic structure and other applications, including the monitoring of the genetic effects of the habitat fragmentation of *C. similis*. These may also help to determine and define valuable targets or distinct units for conservation and for management plans of Korean freshwater crayfish.

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References

Ahn DH, Kawai T, Kim SJ, Rho HS, Jung JW, Kim W, Lim BJ, Kim MS, Min GS. 2006. Phylogeny of northern hemisphere freshwater crayfishes based on 16S rRNA gene analysis. Korean J Genetic. 28:185–192.

Buckley FM, Pollett PK. 2010. Limit theorems for discrete-time metapopulation models. Probability Surveys. 7:53–83.

Crandall KA. 1997. Genetic variation within and among crayfish species. Freshwater Crayfish. 11:135–145.

Crandall KA, Robison HW, Buhay JE. 2009. Avoidance of extinction through nonexistence: the use of museum specimens and molecular genetics to determine the taxonomic status of an endangered freshwater crayfish. Conserv Genet. 10:177–189.

Edgerton BF, Henttonen P, Jussila J, Mannonen A, Paasonen P, Taugbol T, Edsman L, Souty-Grosset C. 2004. Understanding the causes of disease in European freshwater crayfish. Conserv Biol. 18:1466–1474.

Edsman L, Farris JS, Kallersjo M, Prestegaard T. 2002. Genetic differentiation between noble crayfish, *Astacus astacus* (L.), populations detected by microsatellite length variation in the rDNA ITS1 region. B Fr Peche Piscic. 367:691–706.

Gherardi F, Souty-Grosset C, Reynolds J. 2003. Understanding and managing biodiversity in relation to native crayfish populations in Europe. B Fr Peche Piscic. 370: 7–14.

Gouin N, Grandjean F, Souty-Grosset C. 2000. Characterization of microsatellite loci in the endangered freshwater crayfish *Austropotamobius pallipes* (Astacidae) and their potential use in other decapods. Mol Ecol. 9:636–637.

Gouin N, Grandjean F, Souty-Grosset C. 2006. Population genetic structure of the endangered crayfish *Austropotamobius pallipes* in France based on microsatellite variation: biogeographical inferences and conservation implications. Freshwater Biol. 51:1369–1387.

Hammond RL, Saccheri IJ, Ciofi C, Coote T, Funk SM, McMillan WO, Bayes MK, Taylor E, Bruford MW. 1998. Isolation of microsatellite markers in animals. In: Karp A, Isaac PG, Ingram DS, editors. Molecular tools for screening biodiversity. Weinheim: Chapman & Hall. p. 279–285.

Jensen JL, Bohonak AJ, Kelley ST. 2005. Isolation by distance, web service. BMC Genet. 6:13.

Kawai T, Min GS. 2005. Re-examination of type material of *Cambaroides similis* (Koelbel, 1892) (Decapoda: Cambaridae) with a lectotype designation, re-description, and evaluation of geographical variation. Proc Biol Soc Wash. 118:777–793.

Kim HS. 1977. Illustrated encyclopedia of fauna and flora of Korea Vol. 19. Macura. Seoul: Ministry of Education & Samhwa Publishing Co. p. 350–355.

Kim S, Kim YH, Yoon JM. 2006. Genetic variation in geographic crayfish (*Cambaroides similis*) populations. J Fish Pathol. 19:141–153.

Li Q, Park C, Endo T, Kijima A. 2004. Loss of genetic variation at microsatellite loci in hatchery strains of the Pacific abalone (*Haliotis discus hannai*). Aquaculture. 235:207–222.

Lodge DM, Taylor CA, Holdich DM, Skurdal J. 2000. Nonindigenous crayfishes threaten North American freshwater biodiversity: lessons from Europe. Fisheries. 25:7–20.

Rice WR. 1989. Analyzing tables of statistical tests. Evolution. 43:223–225.

Rousset F. 2008. GENEPOP '007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour. 8:103–106.

Rozen S, Skaletsky H. 2000. Primer 3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, editors. Bioinformatics methods and protocols: methods in molecular biology. Totowa (NJ): Humana Press. p. 365–386.

Schuelke M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol. 18:233–234.

Taylor CA. 2002. Taxonomic and conservation status of native crayfish stocks. In: Holdich DM, editor. Biology of freshwater crayfish. Nottingham: Blackwell Science. p. 236–257.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes. 4:535–538.

Versteegen M, Lawler S. 1996. Population genetics of the Murray River crayfish *Euastacus armatus*. Freshwater Crayfish. 11:146–157.

Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution 38:1358–1370.

Yue GH, Li J, Bai Z, Wang CM, Feng F. 2010. Genetic diversity and population structure of the invasive alien red swamp crayfish. Biol Invasions. 12:2697–2706.

Zaccara S, Stefani F, Crosa G. 2005. Diversity of mitochondrial DNA of the endangered white-clawed crayfish (*Austropotamobius italicus*) in the Po River catchment. Freshwater Biol. 50:1262–1272.