Introduction

Microsatellite DNA, short tandem repeats (STRs) of 1–6 nucleotide motifs, is widely used as a convenient tool in population genetics, because of its high polymorphisms, presumed neutrality and co-dominance (Selkoe & Toonen 2006). Microsatellite DNA has been typically used in investigating evolution, phylogeny, population structure and kinships of native species. Recently, microsatellite analysis has also been used in defining eradication units of invasive species (Robertson & Gemmell 2004, Abdelkrim et al. 2005, Rollins et al. 2009, Guillemaud et al. 2010).

The signal crayfish *Pacifastacus leniusculus* (Dana), native to northwestern North America, has been introduced to Europe and Japan for aquaculture purposes. This large omnivore, which grows up to about 15 cm in total length, is known to have strong negative impacts on native ecosystems via predation and ecosystem engineering (e.g. Holdich 1999, Nyström 1999, Usio et al. 2006, 2009). In Japan, *P. leniusculus* has replaced the Japanese crayfish *Cambaroides japonicus* (De Haan) in many Hokkaido lakes and rivers (Kawai et al. 2002) through predation (Nakata & Goshima 2006) and/or competition for shelter (Usio et al. 2001, Nakata & Goshima 2003). In addition, *P. leniusculus* is known to act as a vector for the crayfish plague fungus, *Aphanomyces astaci* (Schikora) that has decimated a number of native crayfish populations in Europe (Dieguez-Uribeondo 2006). Given this background, *P. leniusculus* was designated an Invasive Alien Species (IAS) by the Ministry of the Environment of Japan in 2006. Under the
materials and methods

On five occasions between 1926 and 1930, *P. leniusculus* was introduced to Japan from the Columbia River basin in the United States (Usio et al. 2007). *Pacifastacus leniusculus* was initially distributed to fisheries experimental stations of 29 prefectures, after which these crayfish were introduced to several natural systems including Lake Mashu in Hokkaido (476 individuals were introduced in 1930), Tankai Reservoir (30 individuals were introduced in 1926) and two other ponds/lakes in Shiga Prefecture and either lentic or lotic systems in Fukui Prefecture and Tokyo. Although some of these initial *P. leniusculus* populations, i.e. Fukui, Tokyo and Shiga (other than Tankai Reservoir) populations, disappeared soon after introductions, the Hokkaido populations have gradually expanded their distribution, especially in eastern Hokkaido since the 1970s (Hiruta 1998, Usio et al. 2007). Despite that the *P. leniusculus* populations from the initial introductions have largely disappeared from Honshu, at present feral *P. leniusculus* populations can be found in three localities from Fukushima Prefecture (Lakes Hibara, Onogawa and Akashina) and one locality each from Nagano (Irrigation stream in Akashina), Shiga (Tankai Reservoir basin) and Chiba Prefectures (Tone River basin) (Usio et al. 2007, Nakata et al. 2010). Although there is no official record of crayfish introductions in Akashina, local residents of the township have seen *P. leniusculus* around the Sai River for decades, suggesting that *P. leniusculus* was possibly introduced to Akashina in 1926–30 (Usio et al. 2007, Kawai & Nakata 2009). On the other hand, *P. leniusculus* was found from the three Fukushima lakes around 1998 and the Tone River basin in 2009 (Usio et al. 2007, Nakata et al. 2010). The result of an analysis of ectosymbiont brachiobdellid annelids suggested that the Tone River population was introduced from Hokkaido (Nakata et al. 2010).

In the present study, 212 individuals of *P. leniusculus* were collected from seven localities in Hokkaido and Honshu, Japan (Table 1 and Fig. 1). In our population analyses, the term “sample” is hereafter used to indicate a mass of representative individuals from each locality examined in the present study. Crayfish were collected by hand or using baited Gee-minnow traps. A small piece of muscle tissue was isolated from a cheliped or pleopod of each crayfish; the muscle tissue was fixed in 99% ethanol at room temperature until DNA extraction. Genomic DNA was extracted from 20–50 mg of muscle tissue with a Pure Gene Kit (Qiagen). Genomic DNA was dissolved in 200 μL Tris-EDTA (pH 8.0) or ED buffer (Qiagen).

To construct an enriched microsatellite library, we have basically followed the procedure described in Hamilton et al. (1999). Genomic DNA of one individual from Lake Mashu was digested with the restriction enzymes *RsaI* and *DraI* (TaKaRa), and ligated to double-stranded SNX linkers (SNXF, 5′-CTA AGG CCT TGC TAG CAG AAG C-3′, and SNXR, 5′-GCT TCT GCT AGC GCC TTA GAA C-3′). Portion of each library was isolated from a cheliped or pleopod of each crayfish and used in the population genetic analyses.

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Table 1. Information of the invasive signal crayfish (*Pacifastacus leniusculus*) used in the population genetic analyses.

| Sample name | Number of individuals | Sampling date (Month, Year) | Sampling locality | Latitudes, Longitudes | Year of introduction | Year of discovery |
|-------------|-----------------------|----------------------------|-------------------|----------------------|---------------------|------------------|
| Mashu       | 49                    | Sep. 2005                  | Lake Mashu, Hokkaido | 43°36.36′N, 144°33.36′E | 1930†               | 1930†            |
| Akan        | 18                    | Oct. 2007                  | Lake Akan, Hokkaido | 43°25.48′N, 144°08.24′E | no records         | 1970s*           |
| Toya        | 32                    | Sep. 2008                  | Lake Toya, Hokkaido | 42°34.12′N, 140°49.48′E | no records         | 2005†            |
| Iwabokki    | 30                    | Sep. 2006                  | Iwabokki, Kushiro River, Hokkaido | 43°04.48′N, 144°25.48′E | no records         | 1970s*           |
| Akashina    | 32                    | Oct. 2006                  | Irrigation stream in Akashina, Nagano | 36°21.00′N, 137°55.12′E | no records*        | 1999†            |
| Tankai      | 47                    | Oct. 2006                  | Inlet stream of Tankai Reservoir, Shiga | 35°27.00′N, 135°59.24′E | 1926†               | 1926†            |
| Tone        | 4                     | Sep.–Oct. 2009             | Tone River, Chiba | 35°49.48′N, 140°13.48′E | no records         | 2009†            |

* No official record of introduction exists but crayfish were probably introduced in 1926–30 (see text).
† Table 1 in Usio et al. (2007).
‡ Nakata et al. (2010).
AA-3'). Linker-ligated DNA was denatured, hybridized to biotinylated microsatellite oligonucleotide mix probes containing (CA)$_{10}$, (GA)$_{10}$, (GAT)$_{10}$ and (CAC)$_{10}$ at 60°C, and enriched with streptavidin magnetic particle beads (Dynabeads Streptavidin, Dynal). The captured fragments containing short tandem repeats were again amplified in PCR with SNXF as a primer. The enriched procedure was carried out twice, and the final PCR product was cloned with the TOPO TA Cloning Kit (Invitrogen). We picked up 300 clones and sequenced the insertions with universal M13 forward and reverse primers using the BigDye Cycle Sequencing Kit version 3.1 (Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The PCR primers were designed for unique loci of STRs with appropriate flanking regions using the Genetyx Software (Genetyx Corporation). The utility of the primer sets for PCR amplification was tested using the genome of eight individuals from Mashu, and forward primers of well-amplified loci were fluorescence-labeled and used in population genetic analyses. Polymerase chain reactions (PCR) were carried out with a 20μL reaction mixture containing 2μL of 10×PCR buffer (Sigma), 1.5μM of MgCl$_2$, 0.5μM of unlabeled (reverse) and labeled (forward) primer, 200μM of each dNTP, 0.5U of Taq DNA polymerase (Sigma) and approximately 50–100 ng of template DNA. The thermal profile included pre-cycling denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at temperature suitable for each locus (Table 2) for 30 s and extension at 72°C for 25 s and post-cycling extension at 72°C for 30 min. The PCR products were electrophoresed on the genetic analyzer with a LIZ-500 size standard (Applied Biosystems), and fragment sizes were estimated with the GeneMapper software (Applied Biosystems). We selected the loci that amplified well for genotyping, and deposited the sequence data in GenBank with accession nos. AB610897–AB610901. The primer sets for these loci were used for further analyses.

We first confirmed the effectiveness of the newly characterized microsatellite markers. We used samples from relatively old populations (i.e. Akashina, Tankai and Mashu), because recently established populations may not have reached genetic equilibria such as Hardy-Weinberg equilibrium (HWE) and linkage equilibrium, that are premised on a large and stable population, and may also have low genetic diversity due to founder effects (Reiland et al. 2002, Jamieson 2010). Scoring error, large allele dropout and the presence of null alleles were checked with the MicroChecker 2.2.3 software (Van Oosterhout et al. 2004), and departure from HWE and linkage disequilibrium (LD) were checked with Genepop on the web (Raymond & Rouset 1995, Rouset 2008).

After checking for the validity of the markers, the number of alleles and expected and observed heterozygosities for all samples were calculated with Genepop on the web. Deviations from HWE and LD between loci were also tested with the same software. Allelic richness corrected for sample size was quantified by FSTAT version 2.9.4 (Goudet

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**Table 2.** Characteristics of 5 polymorphic microsatellite loci of the invasive *Pacifastacus leniusculus* in Japan.

| Locus name | Primer sequence (5’-3’) | Repeat motif | $Ta$ (°C) | Allele size range (bp) | $N_a$ | $H_o/H_E$ | GenBank accession no. |
|------------|--------------------------|--------------|----------|------------------------|------|-----------|-----------------------|
| Scop1      | F: gccctctgcttactttctcac R: ectagtaggatgtagctcagaa | (CA)$_{25}$ | 56       | 120–164                | 23   | 0.76/0.92 | AB610897             |
| Scop9      | F: gctgaaatggagggatga R: tgtgccttttctaagctgt | (GA)$_{17}$ | 52       | 145–151                | 4    | 0.38/0.55 | AB610898             |
| Scop13     | F: tcaggtgagacatactagt R: tgtgctcttctctcagaa | (GA)$_{17}$CT(CA)$_{26}$ | 53       | 109–119                | 5    | 0.49/0.44 | AB610899             |
| Scop19     | F: ataacggttaaggaaggtg R: gagaaaaaatgattttcagaa | (GA)$_{17}$ | 43       | 255–265                | 6    | 0.25/0.30 | AB610900             |
| Scop31     | F: gatctgacgtcagctct R: ccctgacattctagtagg | (CA)$_{19}$ | 56       | 160–216                | 33   | 0.84/0.94 | AB610901             |

$Ta$=annealing temperature, $N_a$=observed number of alleles, $H_E$=expected heterozygosity and $H_o$=observed heterozygosity.
Population structure and genetic connectivity between localities were assessed in two ways by means of pairwise $F_{ST}$ values calculated by Arlequin 2.0 (Schneider et al. 2000) and Bayesian clustering analysis implemented in STRUCTURE ver. 2.1 (Prichard et al. 2000, Falush et al. 2003). To estimate $F_{ST}$ as an index for genetic differentiation between samples, we employed the methods of Slatkin’s linearized $F_{ST}$ (Slatkin 1995) based on the differentiation of allele frequency, and significant deviation from zero of $F_{ST}$ values was tested at $\alpha=0.01$ after a Bonferroni correction (0.05/5). In the STRUCTURE analysis, likelihoods for each number of assumed source populations ($K$) were calculated from allele frequency data, and each individual was assigned to a plausible population. Each run was performed in the admixture model with a burn-in period of 100,000 simulations followed by 1,000,000 MCMC simulations. For each $K$, 10 iterations were conducted. The most plausible number of $K$ was selected by the largest likelihood value ($\ln P(X|K)$) and the smallest variance of $\ln P(X|K)$. The $Q$ value for each individual indicates the probability that the individual was derived from the assumed population, and the average $Q$ for each sample represented the probability that the sample belonged to or derived from the assumed population.

### Results

We found 31 unique microsatellite loci available to design PCR primers. Five of 31 primer pairs successfully amplified polymorphic PCR products for all samples (Table 2). When all samples were combined, the number of alleles ranged from 4 in Scop9 to 33 in Scop31, and expected heterozygosities ranged from 0.304 in Scop19 to 0.941 in Scop31. On the other hand, the remaining 26 primer pairs were not useful in population genetic analysis; the primers were unable to amplify PCR products or genotyped data showed multi-peak or monomorphy.

In the initial population analysis that tested for marker validity using Akashina, Tankai and Mashu populations, departures from HWE or linkage disequilibrium were not significant in all loci and all pairs of loci after Bonferroni corrections (see Appendix 1). When all samples were considered as a single population, departures from HWE were significant in all loci except Scop13, indicating the existence of hierarchical population structure among the samples. The means of expected heterozygosity for all loci were 0.626, 0.517 and 0.557 in Mashu, Akashina and Tankai, respectively. Scoring error, large allele dropout or null alleles were not evident for all loci in Akashina and Mashu, while the possibilities of null allele at Scop1 and scoring error by stuttering peak at Scop9 were evident in Tankai. Overall, these results indicated enough utility of the five microsatellite markers for further population genetic analyses.

Significant genetic divergence was observed in most pairs of samples (Table 3) except for Mashu versus three samples (Akan, Iwabokki and Tone) and Tone versus four Hokkaido samples (Mashu, Akan, Toya and Iwabokki). These results suggested that genetic connectivity exists between Mashu and other examined samples from Hokkaido except Toya. Also, it was suggested that the population in Tone originated from Hokkaido, not from Akashina or Tankai. The highest $F_{ST}$ was between Toya and Akashina. The $F_{ST}$ of Akashina versus others and Tankai versus others were significantly different from zero, suggesting independence of the populations in Akashina and Tankai from other populations.

In the STRUCTURE analysis, mean likelihood values ($\ln P(X|K)$ in 10 iterations were $-3427$, $-3272$, $-3281$ and $-3330$ for $K=2$, 3, 4 and 5, respectively, suggesting that the candidate $K$ is 3 or 4. Variance of $\ln P(X|K)$ were 285.1 and 462.8 for $K=3$ and 4, respectively, indicating that the most plausible number of populations was three. The $Q$ values identified three clusters of samples: Tankai, Akashina and others (samples from Mashu, Akan, Toya, Iwabokki and Tone; hereafter called “Hokkaido cluster”) (Fig. 2, Table 4). The STRUCTURE analysis distinguished Akashina and Tankai from the Hokkaido cluster more clearly than $F_{ST}$ analysis. The STRUCTURE analysis using only the samples from the Hokkaido cluster suggested that the most plausible $K$ is one, as indicated by both the largest likelihood value and the smallest variance.

### Table 3. Pairwise $F_{ST}$ values between seven signal crayfish (*Pacifastacus leniusculus*) populations in Japan. Bold letters indicate significant $F_{ST}$ values after Bonferroni corrections ($\alpha=0.01$).

|        | Mashu | Akan | Toya | Iwabokki | Akashina | Tankai | Tone |
|--------|-------|------|------|----------|----------|--------|------|
| Mashu  | 0.019 |      |      |          |          |        |      |
| Akan   |       | 0.066|      |          |          |        |      |
| Toya   | 0.010 | 0.054| 0.076|          |          |        |      |
| Iwabokki | 0.079 | 0.115| 0.221| 0.100    |          |        |      |
| Akashina | 0.043 | 0.085| 0.125| 0.056    | 0.147    |        |      |
| Tankai | 0.044 | 0.039| 0.010| 0.043    | 0.183    | 0.114  |      |

1995).
Fig. 2. Population genetic structure of *Pacifastacus leniusculus*, indicated by posterior probability assignment of each individual to three assumed populations. Each vertical bar represents one individual. The lengths of black, white and gray bars in each individual indicate the probability of each individual belonging to the assumed populations, pop1, pop2 and pop3 in Table 4. Akashina and Tankai were strongly characterized with the gray and black bars, while the Hokkaido cluster showed a mixture of white, black and gray.

Table 4. Proportions of assigned membership to each assumed population (pop1–3).

| Sample Name | pop1 | pop2 | pop3 |
|-------------|------|------|------|
| Mashu       | 0.266| 0.524| 0.210|
| Akan        | 0.237| 0.531| 0.232|
| Toya        | 0.189| 0.791| 0.020|
| Iwabokki    | 0.141| 0.519| 0.340|
| Akashina    | 0.023| 0.038| 0.939|
| Tankai      | 0.871| 0.033| 0.096|
| Tone        | 0.222| 0.694| 0.084|

The values of bold letters indicate the highest probability of assignment in each sample. The probabilities of assignment to pop1, 2 and 3 of each individual was indicated by black, white and gray bars, respectively, in Fig. 2.

**Discussion**

Utility of microsatellite markers and population structure of *Pacifastacus leniusculus* in Japan

Although the number of available microsatellites markers was limited, the markers identified significant genetic differentiation among the three *P. leniusculus* clusters: Hokkaido, Akashina and Tankai. It is plausible that the differentiation was caused by a strong founder effect followed by genetic drift in each cluster, especially in Akashina and Tankai, where relatively small numbers of individuals seem to have been introduced (Usio et al. 2007). The results of the present study also confirmed that the recently invaded *P. leniusculus* population in Tone River originated in Hokkaido, supporting the result of the analysis of brachiobdellid annelids by Nakata et al. (2010).

Genetic diversity is generally higher in the source population than in derived, newly founding populations (Reiland et al. 2002, Handley et al. 2007, Jamieson 2010). This may be true even for introduced species as reported in the buff-tailed bumblebee, *Bombus terrestris* (Linnaeus) (Schmid-Hempel et al. 2007) and the European starling, *Sturnus vulgaris* (Linnaeus) (Rollins et al. 2009). Among the samples from Hokkaido, Mashu is considered the oldest population in terms of the year of introduction/discovery followed by Akan, Iwabokki and Toya (Usio et al. 2007). It is therefore plausible to assume that *P. leniusculus* were initially introduced to Lake Mashu and that this population is the source for other populations in Hokkaido. As expected, results of the present study indicated that allelic richness was highest in Mashu and lowest in Toya within the Hokkaido cluster. Expected heterozigosity, however, was highest in Akan. This partial inconsistency in population analysis might be attributed to deficiency of the numbers and qualities of the microsatellite markers and/or small sample sizes (i.e. numbers of individuals in each sample) in the present study. Alternatively, this inconsistency might be attributed to extremely high levels of genetic diversity in Hokkaido samples, possibly resulting from the fact that relatively large numbers (i.e. 476 individuals) were introduced to Lake Mashu (Usio et al. 2007). The derived population(s) may inherit as similarly high levels of genetic diversity as the source population if large numbers of founders colonize new ecosystems and/or if multiple colonization events occur from the source. Note that even the sample from Tone River, recently derived from Hokkaido, showed a comparable level of genetic diversity.

Alien species can have increased genetic diversity in the invaded range following multiple introductions from either the introduced or native range (Roman & Darling 2007, Chen et al. 2010, Kolbe et al. 2004). Perhaps *P. leniusculus* was introduced to natural systems in Hokkaido only once, but its origin might not be from a single native locality. A future phylogeographic study in the native and introduced ranges may provide insights about the number of original localities from which *P. leniusculus* was introduced to Japan. Furthermore, use of additional microsatellite markers and samples from the native range together with a modern analytical method, such as the approximate Bayesian computation method (Guillemaud et al. 2010), may reveal the routes and processes of crayfish invasions. Use of multiple genetic markers such as microsatellites, SNPs and mitochondrial DNA may also provide more accurate genetic structure of *P. leniusculus*.

Proposals for controlling *Pacifastacus leniusculus* in Japan

The present study indicated significant genetic differentiation among the three clusters (Akashina, Tankai and Hokkaido) of the examined *P. leniusculus* samples in Japan, suggesting restricted gene flow or isolation among the clusters. This result indicates that the populations in Akashina and Tankai reservoir can be treated as independent eradication units.

On the other hand, the Hokkaido populations may be dif-
ficult to eradicate, suppress or control because they have already spread rapidly in a broad geographical range, as shown in the present study. High levels of genetic diversity were found in the Hokkaido cluster compared to the Akashina or Tankai clusters, supporting rapid range expansion. High genetic diversity associated with repeated introductions is among the key factors determining successful establishment of alien species (Roman & Darling 2007). Although several exceptions have been reported (e.g. Lindholm et al. 2005), many empirical studies using microsatellite analyses have suggested that high genetic diversity leads to successful range expansions in invasive animals (e.g. Colautti et al. 2005, Azzurro et al. 2006) and plants (e.g. Andreakis et al. 2009, Pàiron et al. 2010).

The *P. leniusculus* population in the Tone River basin appeared to have high genetic diversity equivalent to populations in the Hokkaido clusters. If high genetic diversity determines invasion success (Roman & Darling 2007), the crayfish originating from the Hokkaido cluster have high potential for successful establishment following subsequent invasions. Thus, special attention should be paid to identify their dispersal methods and to prevent further spread.

Our results also provide insights into invasion pathways of *P. leniusculus* in Japan. Although invasion pathways of *P. leniusculus* are largely unknown, our results indicated high genetic diversity in all populations in the Hokkaido cluster, suggesting that the numbers of colonizing individuals were relatively large. Anecdotal evidence suggests that juvenile crayfish have been introduced unintentionally together with aquaculture species such as the Japanese smelt (*Hypomesus japonensis* McAllister) or transplanted macrophytes such as water chestnuts (*Trapa*). Such unintentional introductions have been reported in various aquaculture species including the Pacific oyster, *Crassostrea gigas* (Thunberg) (Miura et al. 2006), Japanese clam, *Ruditapes philippinarum* (Adams & Reeve) (Okoshi 2007), and ayu, *Plecoglossus altivelis* (Temminck & Schlegel) (Takamura 2009). Identifying invasion pathways and dispersal methods of *P. leniusculus* is urgently required, probably through large-scale spatio-temporal crayfish sampling together with finer-scale population genetic analysis. Furthermore, social studies, such as interviews and questionnaires, on the distributions of aquaculture species may also provide additional information on *P. leniusculus* invasion pathways.

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Appendix: The number of alleles (N.A.), allelic richness corrected for n=18 (Ar) and expected and observed heterozygosity (H_e and H_o) at each locus in each sample. * and ** indicate significant deviation from HWE at α<0.01 levels before and after Bonferroni correction. Tone sample was excluded from an Ar estimation, because its sample size (n=4) was too small for this analysis.

|        | Scop1 | Scop9 | Scop13 | Scop19 | Scop31 | mean |
|--------|-------|-------|--------|--------|--------|------|
| Mashu  |       |       |        |        |        |      |
| N.A.   | 15    | 4     | 3      | 5      | 17     | 8.8  |
| Ar     | 11.92 | 3.69  | 2.99   | 3.33   | 12.54  | 6.89 |
| n=49   |       |       |        |        |        |      |
| HE     | 0.904 | 0.569 | 0.439  | 0.302  | 0.914  | 0.626|
| HO     | 0.939 | 0.449 | 0.531  | 0.347  | 0.939  | 0.641|
| Akan   |       |       |        |        |        |      |
| N.A.   | 10    | 2     | 3      | 4      | 14     | 6.6  |
| Ar     | 10.00 | 2.00  | 3.00   | 4.00   | 14.00  | 6.60 |
| n=18   |       |       |        |        |        |      |
| HE     | 0.850 | 0.439 | 0.500  | 0.494  | 0.911  | 0.639|
| HO     | 0.722 | 0.278 | 0.444  | 0.389* | 0.778  | 0.522|
| Toya   |       |       |        |        |        |      |
| N.A.   | 11    | 3     | 3      | 2      | 15     | 6.8  |
| Ar     | 9.88  | 2.62  | 2.98   | 2.00   | 12.54  | 6.00 |
| n=32   |       |       |        |        |        |      |
| HE     | 0.875 | 0.122 | 0.475  | 0.466  | 0.906  | 0.569|
| HO     | 0.593*| 0.125 | 0.531  | 0.156  | 0.781* | 0.437**|
| Iwabokki |      |       |        |        |        |      |
| N.A.   | 4     | 3     | 3      | 3      | 17     | 8.0  |
| Ar     | 12.1  | 2.84  | 2.98   | 2.60   | 12.91  | 6.70 |
| n=30   |       |       |        |        |        |      |
| HE     | 0.890 | 0.503 | 0.387  | 0.263  | 0.920  | 0.593|
| HO     | 0.767 | 0.400 | 0.433  | 0.233  | 0.800  | 0.527|
| Akashina |      |       |        |        |        |      |
| N.A.   | 14    | 4     | 4      | 2      | 15     | 7.8  |
| Ar     | 11.47 | 3.53  | 3.48   | 1.99   | 11.28  | 7.09 |
| n=32   |       |       |        |        |        |      |
| HE     | 0.873 | 0.514 | 0.554  | 0.173  | 0.847  | 0.517|
| HO     | 0.844 | 0.469 | 0.781  | 0.188  | 0.938  | 0.626|
| Tankai |       |       |        |        |        |      |
| N.A.   | 11    | 4     | 4      | 2      | 10     | 6.2  |
| Ar     | 9.45  | 3.86  | 3.37   | 1.97   | 8.59   | 5.45 |
| n=47   |       |       |        |        |        |      |
| HE     | 0.879 | 0.645 | 0.251  | 0.139  | 0.872  | 0.557|
| HO     | 0.660*| 0.446*| 0.277  | 0.149  | 0.787  | 0.463*|
| Tone   |       |       |        |        |        |      |
| N.A.   | 4     | 2     | 2      | 3      | 5      | 4    |
| Ar     | 0.775 | 0.250 | 0.425  | 0.600  | 0.900  | 0.738|
| n=4    |       |       |        |        |        |      |
| HE     | 0.750 | 0.250 | 0.500  | 0.750  | 0.750  | 0.750|
| HO     | 0.764**| 0.377*| 0.491  | 0.245* | 0.844**| 0.544**|
| Total  |       |       |        |        |        |      |
| N.A.   | 23    | 4     | 5      | 6      | 33     | 14.2 |
| Ar     | 9.25  | 0.554 | 0.437  | 0.304  | 0.941  | 0.557|
| n=212  |       |       |        |        |        |      |
| HE     | 0.764**| 0.377*| 0.491  | 0.245* | 0.844**| 0.544**|