

1 Genetic differentiation in the endangered myrmecophilous butterfly *Niphanda fusca*: a
2 comparison of natural and secondary habitats

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17

18 **Abstract**

19 *Niphanda fusca* is an endangered myrmecophilous butterfly inhabiting environments at
20 early stages of succession. Most of its habitats are places where succession is prevented
21 by human activity. In some places, however, *N. fusca* lives in natural semi-bare areas,
22 such as cliffs in mountains or grasslands around volcanos. We investigate the genetic
23 structure of *N. fusca* in Japan and South Korea to address two questions. 1) Are
24 populations in natural environments genetically different from those in secondary
25 environments? and 2) Do populations in natural environments possess greater genetic
26 diversity than those in secondary environments? The AMOVA results indicated that the
27 populations in natural environments and those in secondary environments were
28 differentiated to some extent; however, more than 80% of genetic variation was found to
29 occur within the same habitat type and within each population. We found no differences
30 in genetic diversity between populations in the two environments. At present, we have
31 not found a strong reason to consider populations in the two environments as different
32 evolutionarily significant units. We think it is practical to conserve populations in
33 natural environments at first, because in this case we need not manage habitats to
34 protect *N. fusca*. We have only to inhibit habitat destruction. In contrast, in order to
35 conserve populations in secondary environments, we would have to continue managing
36 the habitats. This is far more difficult than inhibiting habitat destruction.

37

38 **Keywords:** ant, genetic structure, mitochondrial DNA, nuclear DNA, parasite

39

40 **Introduction**

41 Population fragmentation enhances the risk of extinction through loss of
42 genetic diversity via drift, inbreeding, and local adaptation (Frankham et al. 2002).
43 Human activities are one of the greatest threats to population fragmentation of natural
44 organisms (e.g., Laurance et al. 2000; Pimm SL et al. 2014). However, some life history
45 traits of natural organisms would also lead to population fragmentation. To what extent
46 populations experience fragmentation varies depending on the species.

47 The majority of lycaenid butterflies have associations with ants that can be
48 facultative or obligate, and range from mutualism to parasitism (Pierce et al. 2002).
49 Some species are obligate parasites: they live in ant nests where they are fed
50 mouth-to-mouth by the adult ants or eat the ant brood (Pierce et al. 2002). Associations
51 between butterflies and ants have attracted the attention of many biologists because they
52 provide an ideal opportunity to study symbiotic relationships (e.g., Als et al. 2004;
53 Eastwood et al. 2006; Nash et al. 2008). Myrmecophilous butterflies have other notable
54 characteristics. In butterflies that have obligate association with ants, overlapping

55 requirements of suitable host plants and attendant ants can lead to population
56 fragmentation, thus promoting genetic divergence among populations. Such population
57 fragmentation must also increase the risk of local extinction (Frankham et al. 2002). In
58 fact, obligate myrmecophilous butterflies have seriously declined. The best-documented
59 example is the extinction and reintroduction of the large blue *Maculinea arion* in
60 England (Thomas et al. 2009).

61 *Niphanda fusca* is a lycaenid butterfly distributed in Eastern Asia (Fukuda et al.
62 1984). This species is an ant parasite: 1st-2nd instar larvae drink honeydew of aphids on
63 various plants, and 3rd-last instar larvae are brought into the nest of a host ant,
64 *Camponotus japonicas*, and fed mouth-to mouth by the ant (Fukuda et al. 1984; Hojo et
65 al. 2009). The butterfly previously had a wide geographic range throughout the Japan
66 mainland except for Hokkaido, which consisted of many patchy and small habitats.
67 However, it has become extinct in many areas (Mano and Fujii 2009), and is listed as
68 Endangered (facing a high risk of extinction in the wild in the near future) in the Japan
69 Red List (Ministry of the Environment of Japan 2012). The habitat requirement of *N.*
70 *fusca* is relatively specific. *C. japonicas* builds its nests in sunny places (Imai et al.
71 2004), and therefore *N. fusca* also inhabits such places. Of course, *N. fusca* cannot
72 inhabit all the places where *C. japonicas* builds its nests; for instance, it can only inhabit
73 places where there are sufficient aphids. *N. fusca* prefers early stages of succession,
74 such as grasslands or semi-bare areas (Fukuda et al. 1984). In the rainy climate of Japan,
75 most semi-bare areas and grasslands become forests through succession (Kira 1971).
76 Actually, most habitats of *N. fusca* are places where succession is prevented by human
77 activity (Fukuda et al. 1984), such as satoyama: the traditional agricultural landscape of
78 Japan, consisting of a mosaic of patches of forests, grasslands, ponds, and creeks
79 (Washitani 2001). Now, many of these secondary-environment habitats have become
80 unsuitable for *N. fusca*: such secondary environments have been destroyed or are
81 becoming forests because they are not managed now (Mano and Fujii 2009). This
82 declining situation is similar to that of *Maculinea* butterflies, for which habitat
83 management is now actively performed (Thomas et al. 2009; Ugelvig et al. 2011). On
84 the other hand, *N. fusca* also lives in natural semi-bare areas, such as cliffs in mountains
85 or grasslands around volcanos. Since these natural-environment habitats are less
86 affected by human life-style than habitats in secondary environments, they may be
87 stable for much longer periods.

88 From the point of view of conservation strategy, it is easier to protect
89 populations in natural environments than those in secondary environments because the
90 existence of the latter populations depends on changeable human life-style factors that
91 are difficult to control. Moreover, populations in natural environments may be a richer

92 source of genetic diversity than populations in secondary environments because the
93 latter might have often experienced a bottleneck and a founder effect (e.g., DeChaine
94 and Martin 2004; Neve et al. 2009). However, it is possible that *N. fusca* populations in
95 natural environments and those in secondary environments might have been genetically
96 differentiated, and should be treated as different evolutionarily significant units (ESU)
97 (Crandall et al. 2000).

98 In this study, we investigate the genetic structure of *N. fusca* to address two
99 questions. 1) Are populations in natural environments genetically different from those in
100 secondary environments? and 2) Do populations in natural environments possess greater
101 genetic diversity than those in secondary environments? For this purpose, we analyzed
102 the distributions of mitochondrial and nuclear DNA haplotypes of this butterfly in Japan
103 and South Korea.

104

105 **Materials and Methods**

106

107 *Sampling protocol*

108 We collected as samples 189 individuals of *N. fusca* from 21 sites representing the
109 species' geographic range in Japan from 2010 to 2012, and 7 individuals from three
110 sites in South Korea (Namyangju: 4, Yeongwol: 2, Inje: 1) in 2012 (Fig. 1). Samples
111 were preserved in 99% ethanol or acetone at -25°C. Since *N. fusca* is an endangered
112 species in Japan (Ministry of the Environment of Japan 2012), we need to take care not
113 to damage its populations by our sampling. In each sampling site, we selectively
114 collected a few older individuals per site. For additional sampling, we captured a
115 butterfly with an insect net, and cut one middle or hind leg. Then the butterfly was
116 marked with water-insoluble ink to avoid re-sampling, and released. We stopped taking
117 samples when we had collected ten or more butterflies. Therefore, the sample size
118 reflects the population size to some extent.

119

120 *Extraction of genomic DNA, PCR and sequencing*

121 Genomic DNA was extracted from individual thoraces or legs using a DNeasy Blood &
122 Tissue Kit (Qiagen), following the manufacturer's instructions. Fragments of the
123 mitochondrial cytochrome *c* oxidase subunit I (COI) gene were amplified by
124 polymerase chain reaction (PCR) using primer pair Ron
125 (5'-GGATCACCTGATATAGCATTCCC-3') and Nancy
126 (5'-CCCGGTAAAATTTAAATATAAACTTC-3') (Simon et al. 1994). Fragments of the
127 mitochondrial NADH dehydrogenase subunit 5 (ND5) gene were PCR amplified using
128 primer pair V1 (5'-CCTGTTTCTGCTTTAGTTCA-3') and A1

129 (5'-AATATDAGGTATAAATCATAT-3') (Yagi et al. 1999). Fragments of the nuclear
130 elongation factor 1 α (EF-1 α) gene were PCR amplified using ef44
131 (5'-GCYGARCGYGARCGTGGTATYAC-3') and efrCM4
132 (5'-ACAGCVACKGTYTGYCTCATRTC-3') (Monteiro and Pierce 2001).
133 Amplifications were conducted with a 3-min denaturation at 94°C followed by 35 cycles
134 of 0.5 min denaturation at 94°C, 1 min annealing at 50°C for COI and EF-1 α , and 44 °C
135 for ND5, 1.5 min extension at 72°C, and a final 7 min extension at 72°C. We used rTaq
136 or ExTaq DNA polymerase (Takara, Otsu, Japan) in a thermal cycler (Takara, Otsu,
137 Japan). PCR products were cleaned up using ExoSAP-IT (USB Corporation, Cleveland,
138 OH). Cycle sequencing reactions were carried out using a BigDye terminator version
139 3.1 (ABI) using both primers. For EF-1 α , we designed an internal forward primer efNif
140 (5'-TGCCCTGGTTCAAGGGATGG-3') because PCR products were slightly too long
141 (ca. 1000 bp) for cycle sequencing reaction. After removing impurities, the products
142 were sequenced using an ABI 3130xl sequencer (ABI). For COI and ND5, the
143 overlapping region of each strand was used for analyses. For EF-1 α , the
144 non-overlapping region was also used because the sequence data were very clear. For
145 EF-1 α samples that contained multiple heterozygous sites, we performed TA cloning.
146 Haplotype sequences were deposited in DNA Data Bank of Japan (Accession numbers
147 AB844713–AB844726, LC026482–LC026491).

148

149 *Data Analyses*

150 The obtained alignment was straightforward and required no gap filling. Sequences
151 were aligned with Clustal W2 (Larkin et al. 2007). For sampling sites with number of
152 genes ≥ 10 , genetic diversity within the sampling site was estimated by computing
153 haplotype diversity (H) and nucleotide diversity (π) (Nei 1987) using Arlequin 3.5
154 (Excoffier and Lischer 2010). Haplotype diversity is the probability that two randomly
155 sampled alleles are different, while nucleotide diversity is the average number of
156 differences in nucleotides per site between two DNA sequences. The statistical
157 parsimonious network was calculated using TCS version 1.21 (Clement 2000). The
158 network was subsequently drawn by hand (Fig. 2a,b). The data from the two
159 mitochondrial genes were concatenated for these analyses.

160 We performed an analysis of molecular variance (AMOVA: Excoffier et al.
161 1992) implemented in Arlequin 3.5 (Excoffier and Lischer 2010) to separate *N. fusca*
162 genetic variation into components attributable to differences among the hierarchical
163 groups (habitat type: natural or secondary environments) (Φ_{CT}), among sampling sites
164 within each habitat type (Φ_{SC}), and among sampling sites across the *N. fusca*
165 distributional range (Φ_{ST}). We performed 1000 permutations under the null hypothesis

166 of panmixia to test significance. Since only Eastern Japan contains natural-environment
167 habitats (Fig. 1), AMOVA including all the Japanese populations might confuse effects
168 of habitat type and geographic signals. Therefore, we also performed AMOVA
169 including only sampling sites in Eastern Japan (sampling sites 1~14). In addition, there
170 may be better clustering of sampling sites than habitat type. A spatial AMOVA using
171 SAMOVA ver. 2.0 (Dupanloup et al. 2002) was performed to identify the genetic
172 cluster of sampling sites that maximized the F_{CT} value. We performed 100 simulated
173 annealings for $K = 2$ to $K = 20$ partitions of sampling sites. Isolation by distance was
174 tested by the Mantel test performed on matrices of pairwise geographic distances
175 (ground distances) and pairwise F_{ST} values. We performed 1000 permutations to test
176 significance using Arlequin 3.5 (Excoffier and Lischer 2010).

177 AMOVA, SAMOVA and the Mantel test were applied to Japanese samples
178 because the aim of this study was to analyze the genetic structure of *N. fusca* of the
179 Japanese archipelago. For these analyses, a combined dataset of mitochondrial DNA
180 and EF-1 α as separate loci was used

181

182 **Results**

183 We obtained the DNA sequences of 388 bp of the COI gene, 614 bp of the ND5 gene,
184 and 654 bp of the EF-1 α gene. In the data set of the two mitochondrial genes, we found
185 11 polymorphic sites (6 in COI and 5 in ND5) leading to 12 haplotypes (7 in COI and 6
186 in ND5) (Fig. 2a). The 7 alleles in the COI gene were named a~g, and the 6 alleles in
187 the ND5 genes were named 1~6 (Fig. 2a). For the EF-1 α gene, we found 7 polymorphic
188 sites leading to 8 haplotypes (Fig. 3a)

189 Mitochondrial genetic diversity indices for each sampling site in the Japanese
190 archipelago are presented in Table 1. Both haplotype diversity (H) and nucleotide
191 diversity (π) were 0 (consisting of a single haplotype) for 11 out of 15 sampling sites
192 with number of samples ≥ 10 . Haplotype diversity was 0.33-0.6, and nucleotide
193 diversity was 0.00038-0.00098, for the remaining 4 sampling sites, which contained 2 to
194 3 haplotypes. Of 5 natural-environment sampling sites, 3 contained a single haplotype.
195 Of 10 secondary-environment sampling sites, 8 contained a single haplotype. A
196 difference in haplotype diversity was not found between these two types of sampling
197 sites (Wilcoxon rank sum test: $W = 32.5$, $P = 0.503$). Also, a difference in nucleotide
198 diversity was not found between the two types of sampling sites (Wilcoxon rank sum
199 test: $W = 34.5$, $P = 0.333$).

200 Genetic diversity indices of EF-1 α for each sampling site are presented in
201 Table 1. Both haplotype diversity (H) and nucleotide diversity (π) were 0 (samples all
202 had a single haplotype) for 5 out of 19 sampling sites with number of samples ≥ 5 .

203 Haplotype diversity was 0.1-0.63, and nucleotide diversity was 0.00015-0.0015 for the
204 remaining 14 sampling sites, which contained 2 to 3 haplotypes. Of 5
205 natural-environment sampling sites, 2 contained a single haplotype. Of 14
206 secondary-environment sampling sites, 3 contained a single haplotype. A difference in
207 haplotype diversity was not found between these two types of sampling sites (Wilcoxon
208 rank sum test: $W = 28.5$, $P = 0.575$). Also, a difference in nucleotide diversity was not
209 found between these two types of sampling sites (Wilcoxon rank sum test: $W = 29.5$, P
210 $= 0.64$).

211 The AMOVA results were significant, indicating that 15.11% of the *N. fusca*
212 genetic variation could be explained by the habitat type, and 52.93% of the genetic
213 variation could be attributed to differences among sampling sites within each habitat
214 type (Table 2). The result of AMOVA including only Eastern Japanese populations
215 were also significant; however, differentiation between the habitat types was not found
216 (Table 2). The SAMOVA results showed that our genetic data were best explained by
217 assuming the existence of 19 groups. One group consisted of the populations in Oguni,
218 Otari, and Higashiizu, which contained exactly the same haplotypes of mitochondrial
219 and nuclear genes (Fig. 2b,3b). Each of the remaining sampling sites contained a
220 different group.

221 The correlation between geographic distance and F_{ST} among Japanese sampling
222 sites was significant according to the Mantel test ($P = 0.027$).

223

224 **Discussion**

225 Genetic diversity indices for each sampling site of *N. fusca* are lower than those of other
226 butterflies (de Jong et al. 2011; Sielezniew et al. 2011; Downey and Nice 2013; Bossart
227 and Antwi 2013; Sakamoto et al. 2015), and are similar to those of introduced butterfly
228 populations (Wu et al. 2010). Some butterflies that parasitize ant nests also exhibit low
229 genetic diversity (Ugelvig et al. 2011; Sielezniew et al. 2012; Pellissier et al. 2012). In
230 parasitic butterflies, overlapping requirements of suitable host plants and attendant ants
231 would lead to population fragmentation, reduced effective population size, and
232 consequently, decrease of genetic diversity. One of our working hypotheses is that
233 populations in natural environments would contain more genetic diversity than
234 populations in secondary environments because the latter would have experienced more
235 bottleneck and founder effects. However, differences in genetic diversity were not
236 found here between natural-environment sampling sites and secondary-environment
237 sampling sites. At this stage, therefore, the low genetic diversity in *N. fusca* should be
238 attributed to their obligate parasite life history, rather than habitat loss caused by change
239 of human life-style.

240 Habitat type explained 15.11% of *N. fusca* genetic variation. This result seems
241 to indicate that populations in natural environments and those in secondary
242 environments are somewhat differentiated. However, all five natural-environment
243 habitats studied here were located in Eastern Japan (Fig. 1), and the AMOVA results
244 may reflect a local population divergence. In fact, when we performed AMOVA
245 including only sampling sites in Eastern Japan, habitat type could not explain *N. fusca*
246 genetic variation (Table 2).

247 52.93% of the genetic variation was found to arise within the same habitat type,
248 indicating that factors other than habitat type have a major effect on *N. fusca* geographic
249 variation. In the present case, the result of the Mantel test was significant, indicating
250 that geographic distance plays a role. Potential factors that could have caused the
251 geographic variation include an effect of symbiosis. Host ants may play a role in the
252 divergence of butterflies (Als et al. 2004; Eastwood et al. 2006). In addition, it is known
253 that symbiotic bacteria such as *Wolbachia* can also affect the genetic structure of their
254 host insects through a selective sweep (e.g. Narita et al. 2006; Graham and Wilson
255 2012; Sielezniew et al. 2012). Further research is needed to clarify these effects.

256 Our data did not provide a strong reason to consider natural-environment
257 populations and secondary-environment populations as different ESUs. The SAMOVA
258 results indicated that *N. fusca* cannot be clustered genetically in a few large groups,
259 although each sampling site exhibits genetic differences. Perhaps this is because the
260 genetic differentiation of each sampling site is limited. However, it should be noted that
261 this study used only genetic markers. Phenotypic adaptations may occur in both types of
262 environments.

263 *N. fusca* is already endangered in Japan (Ministry of Environment of Japan
264 2012), and its conservation must be initiated as soon as possible. At present, we think it
265 is practical to conserve the butterflies in natural environments at first, because in that
266 case, we need not manage habitats to protect *N. fusca* for the time being. We have only
267 to prevent habitat destruction. In contrast, in order to conserve the butterflies in
268 secondary environments, we would have to continue managing the habitats. This is far
269 more difficult than preventing habitat destruction because it requires that we place
270 sufficient new value on the traditional environmental management to continue it
271 (Washitani 2001). It is fortunate for *N. fusca* that there exist natural-environment
272 habitats, although their number is limited (Fig. 1). In addition, we were able to collect
273 ten or more samples in all of the five natural-environment sampling sites, while we
274 could not collect ten samples in six secondary-environment sampling sites (Table 1),
275 suggesting that the population sizes of natural-environment habitats are relatively larger.
276 Some butterfly species living only in secondary grasslands in Japan, such as

277 *Shijimiaeoides divines*, *Melitaea protomedia*, and *Fabriciana nerippe*, are experiencing
278 much more severe declines because there are no remaining habitats for them when
279 secondary grasslands are abandoned or destroyed (Nakamura 2011).

280 Of course, we do not think that it is impossible to maintain habitats in
281 secondary environments. Conservation of secondary-environment habitats could be
282 successful in habitats that contain many endangered species and that are considered
283 valuable by many people.

284

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293

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Table 1 The mitochondrial and nuclear gene diversity index of each sampling site. Number of individuals, haplotype diversity (H) \pm SD and nucleotide diversity (π) \pm SD of each site are shown. Location numbers are identical to those in Fig.1

Location	Habitat	Number of individuals	mitochondrial DNA		nuclear DNA	
			H (\pm SD)	π (\pm SD)	H (\pm SD)	π (\pm SD)
1. Oguni	secondary	7			0	0
2. Uonuma	natural	10	0.4667 \pm 0.1318	0.000466 \pm 0.0005	0.6158 \pm 0.077	0.001086 \pm 0.000951
3. Shirosato	secondary	13	0	0	0.1732 \pm 0.1009	0.000265 \pm 0.000406
4. Otari	secondary	10	0	0	0	0
5. Fujikawaguchiko	natural	11	0	0	0.4978 \pm 0.1022	0.000872 \pm 0.000793
6. Higashiizu	secondary	3				
7. Nakatsugawa	secondary	5			0.5333 \pm 0.0947	0.000815 \pm 0.000833
8. Ohno	natural	10	0	0	0	0
9. Higashiohmi	natural	11	0.3273 \pm 0.1533	0.00098 \pm 0.000808	0	0
10. Miyagawa	natural	11	0	0	0.1732 \pm 0.1009	0.000265 \pm 0.000406
11. Nara	secondary	10	0	0	0.3368 \pm 0.1098	0.000515 \pm 0.000596
12. Totsugawa	secondary	10	0	0	0	0
13. Sanda	secondary	10	0	0	0.1000 \pm 0.0880	0.000153 \pm 0.000303
14. Toyooka	secondary	13	0.3846 \pm 0.1321	0.000384 \pm 0.000433	0.5942 \pm 0.0537	0.001019 \pm 0.000904
15. Maniwa	secondary	11	0	0	0.6277 \pm 0.0602	0.001119 \pm 0.000965
16. Nishinoshima	secondary	12	0	0	0.5543 \pm 0.0872	0.001097 \pm 0.000949

17. Akiolta	secondary	5			0.6000±0.1305	0.001529±0.001267
18. Umi	secondary	10	0.6 ± 0.1305	0.000665 ± 0.000628	0.5053±0.0560	0.000773±0.000763
19. Higashisonogi	secondary	5			0.5333±0.0947	0.000815±0.000833
20. Takamori	secondary	10	0	0	0.1000±0.0880	0.000153±0.000303
21. Tarumizu	secondary	2				

Table 2 Analyses of molecular variance (AMOVA) for grouping by habitat type

	d.f.	Sum of Squares	Variance component	Percentage of variation	Fixation Index	P
Japan						
among G	1	19.95	0.0867 Va	15.11	Φ_{CT} : 0.15113	0.03128
among P	19	105.419	0.31058 Vb	52.93	Φ_{SC} : 0.62355	< 0.00001
within P	347	65.063	0.18750 Vc	31.96	Φ_{ST} : 0.68044	< 0.00001
Easten Japan						
among G	1	17.204	0.0751Va	12.56	Φ_{CT} : 0.12556	0.09286
among P	12	84.552	0.37995Vb	63.52	Φ_{SC} : 0.76077	< 0.00001
within P	244	34.914	0.14309Vc	23.92	Φ_{ST} : 0.72642	< 0.00001

Fig. 1 The locations of the sampling sites. Open circles indicate natural environments, and filled circles indicate secondary environments. Sampling sites are indicated by the city or town name. 1. Oguni, 2. Uonuma, 3. Shiroshato, 4. Otari, 5. Fujikawaguchiko, 6. Higashiizu, 7. Nakatsugawa, 8. Ohno, 9. Higashiohmi, 10. Miyagawa, 11. Nara, 12. Totsugawa, 13. Sanda, 14. Toyooka, 15. Maniwa, 16. Nishinoshima, 17. Akiohta, 18. Umi, 19. Higashisonogi, 20. Takamori, 21. Tarumizu, 22. Namyangju, 23. Yeongwol, 24. Inje

Fig. 2 (a) A haplotype network of mitochondrial genes. Each node in the haplotype network represents a single nucleotide change and each branch represents a single mutational step. The alphabetical part of the haplotype names indicates the COI allele, and the numerical part of the haplotype names indicates the ND5 allele. Circle areas in the haplotype network are proportional to observed numbers of haplotype copies present in all the samples.

(b) Distribution of mitochondrial haplotypes Haplotypes shared by at least two sampling sites are indicated by the same pattern, and unique haplotypes present only in one specific site are indicated by the haplotype names.

Patterns and names for each haplotype are the same in **(a)** and **(b)**.

Fig. 3 (a) A haplotype network of the nuclear gene.

(b) Distribution of nuclear haplotypes.

Fig. 1

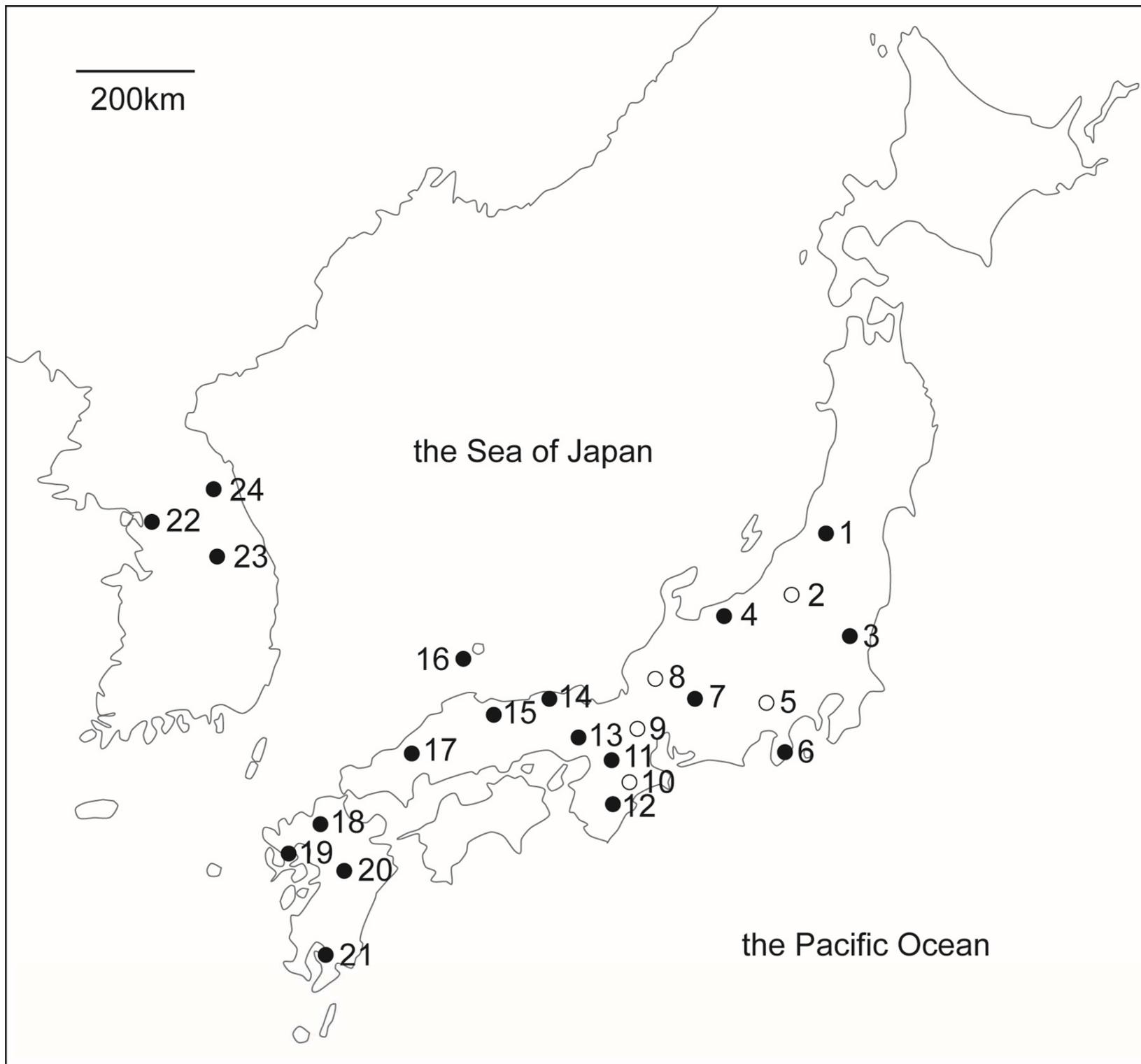


Fig. 2(a)

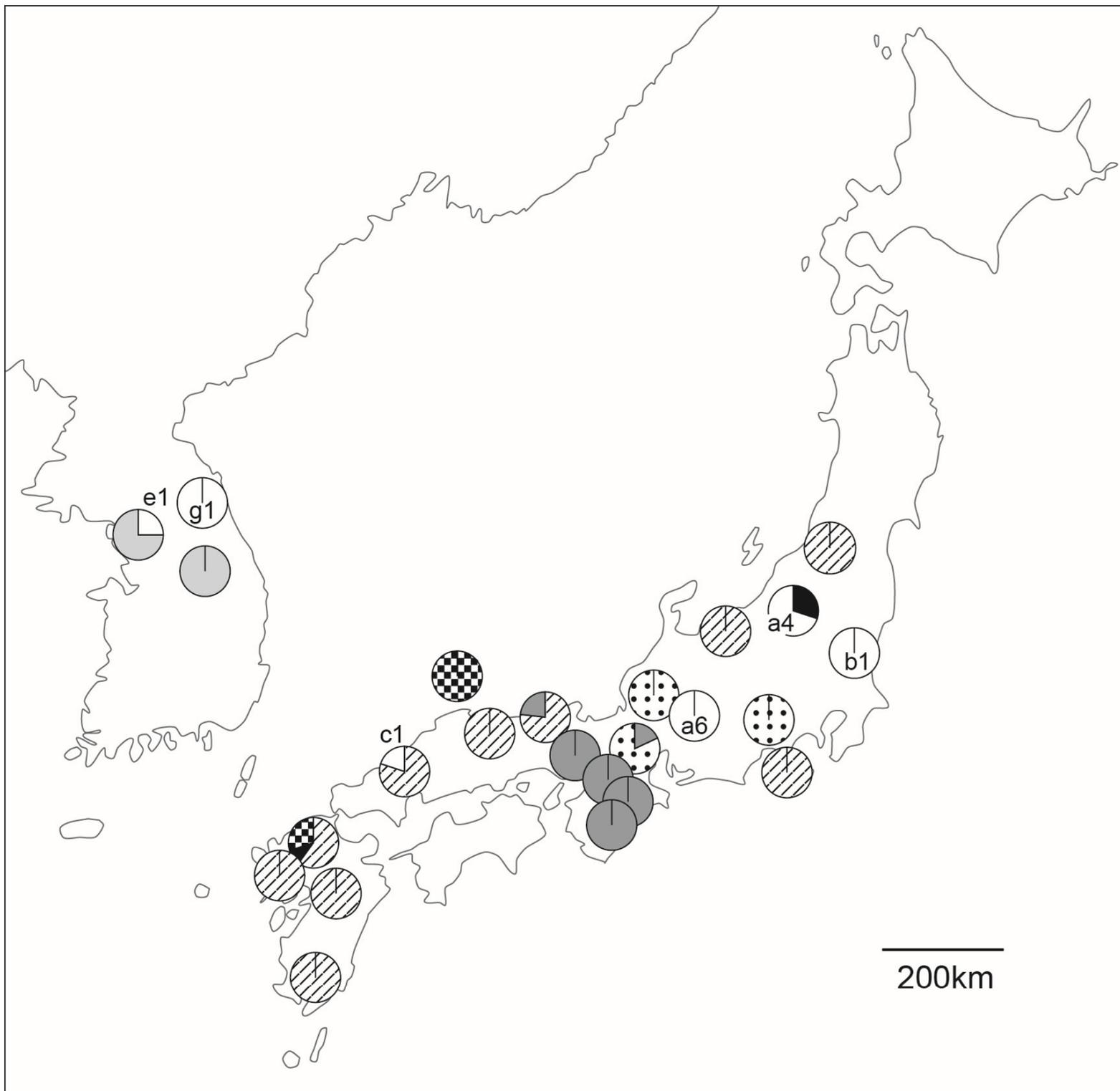


Fig. 3(a)

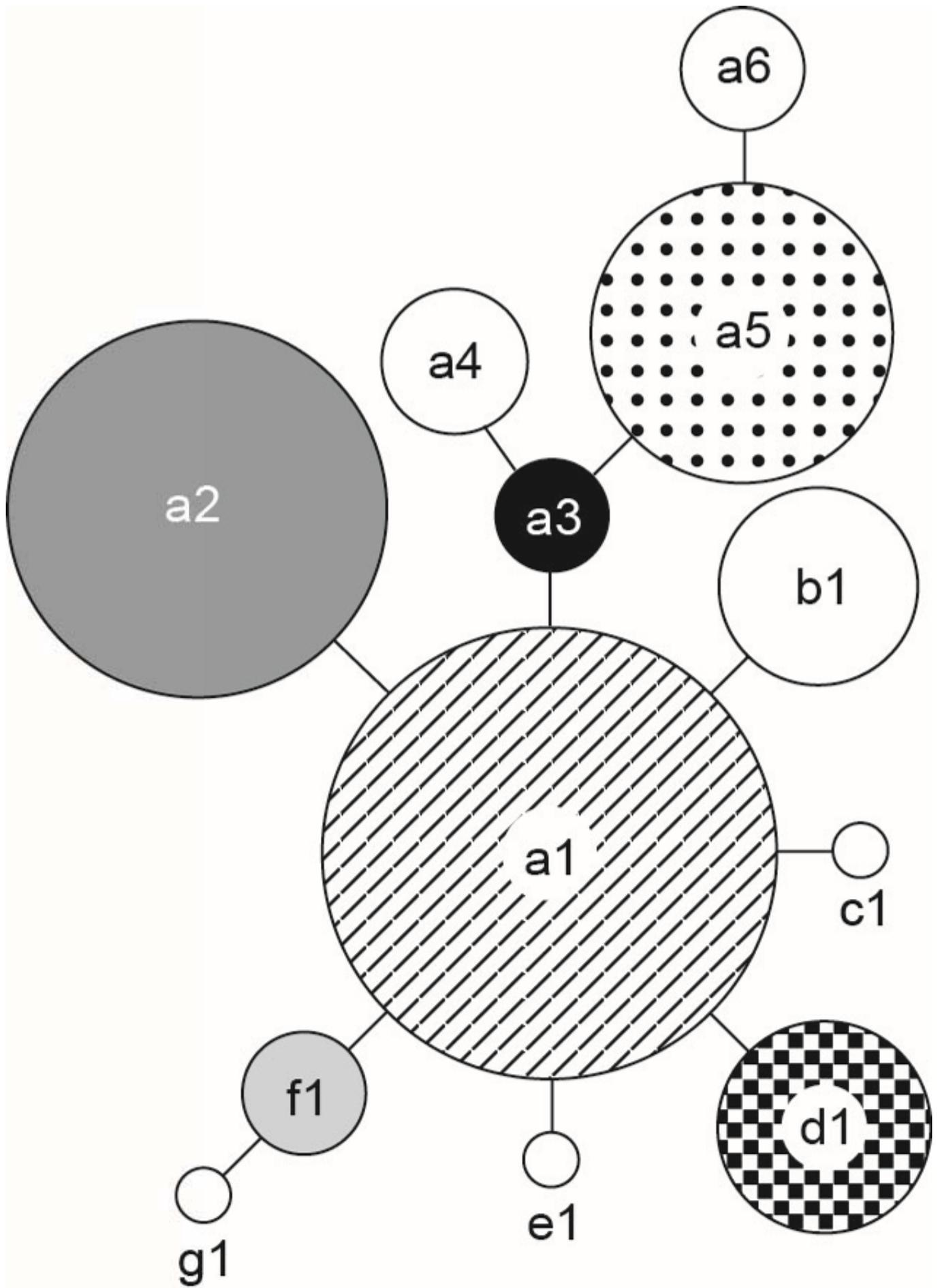


Fig. 3(b)

