

## Population genetic analysis of a highland DNA clade in the red ant *Myrmica kotokui* Forel 1911 (Formicidae, Myrmicinae)

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### Abstract

Japanese mountain ecosystems may face a crisis of extinction owing to habitat fragmentation and population isolation due to global warming caused by human activities. In this study, we studied highland clades of *Myrmica kotokui* by phylogenetic and population genetic techniques to investigate population fragmentation and isolation in mountain ecosystems of the Japan Alps. We reconstructed a molecular phylogeny from DNA sequences of the mitochondrial *cytochrome oxidase I (COI)* gene of highland clades of *Myrmica kotokui* specimens collected from six mountain regions in the Japan Alps. Then, we calculated two genetic diversity indexes (haplotype and nucleotide diversity) for every mountain population and compared the results among populations. Haplotype diversity values were high in all mountain regions, but nucleotide diversity values were lower in two southern mountain regions (the Chuo and Minami Alps) than in the other regions. This pattern of high haplotype and low nucleotide diversity suggests that a historical population bottleneck, perhaps, a few million years ago was followed by rapid population growth and accumulation of mutations. This result suggests that the decline of nucleotide diversity in the southern mountain regions might not be associated with recent global warming caused by human activities.

**Key words:** *COI* gene, genetic diversity, phylogenetic analysis, Japan Alps

### Introduction

Since the end of the last glacial period, mountain organisms with distributions restricted to highlands have become evolutionarily isolated, and endemic mountain ecosystems have become established in each mountain regions. However, these unique mountain ecosystems may face a crisis of extinction as a consequence of global warming caused by human activities, owing to habitat fragmentation and population isolation. The degree to which local extinctions have occurred in an ecosystem can be estimated by examining the genetic diversity of a member of the ecosystem (Manel *et*

*al.*, 2003), because genetic diversity correlates with population size and is decreased when habitats become fragmented and populations become spatially isolated (Young *et al.*, 1996). Thus, estimations of genetic diversity can be used to help identify endangered ecosystems. Detailed information on the genetic diversity of animals in the Japan Alps, however, is not available.

Ants have multiple ecological functions in terrestrial ecosystems, including predation, seed dispersal, and plant protection (Hölldobler and Wilson, 1990). Thus, ants are keystone species in terrestrial ecosystems, and their genetic diversity may thus reflect the degree of habitat fragmentation

and population isolation in ecosystems.

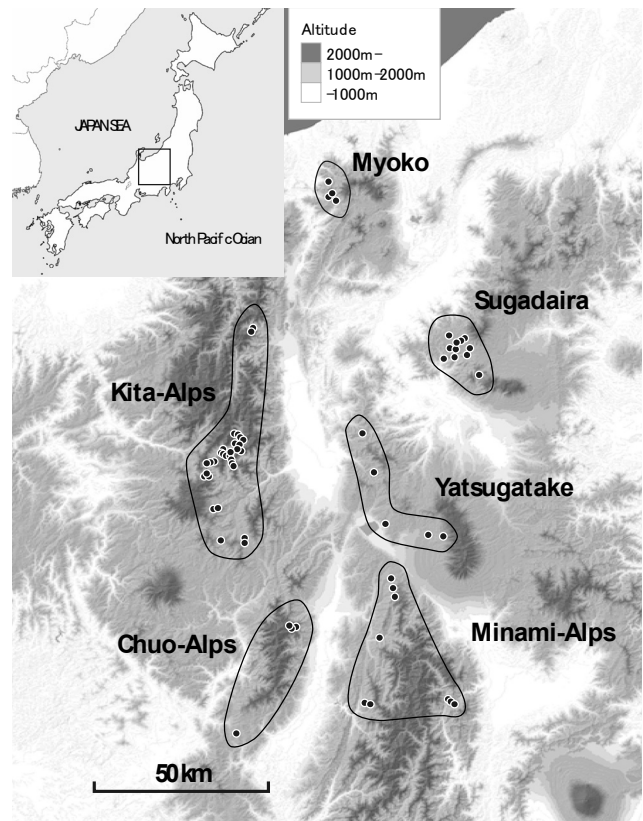
*Myrmica kotokui* (Myrmicinae) is a common species of red ant in far eastern Asia that is distributed in the Russian Far East, the Korean Peninsula, northeastern China, and the Japanese Archipelago (Radchenko and Elmes, 2010). In the Japan Alps of central Honshu, this ant species is distributed over a broad elevational range, from approximately 1000 to 2000 m above sea level (a.s.l.) (Togai *et al.*, 2012). Ueda *et al.* (2012) investigated the spatial patterns of genetic variation in *M. kotokui* by reconstructing a molecular phylogeny of mitochondrial and nuclear gene sequences using specimens collected along an elevation gradient from 900 to 1800 m a.s.l. in the Japan Alps. This phylogeny revealed four highly differentiated clades (L1–L4) that showed distributional differences by elevation. Furthermore, ecological and morphological characters, such as habitat and nesting microhabitats and the head width of queens, are also differentiated among the clades (Ueda *et al.*, 2013). Among these, L3 is a highland clade that is distributed at higher elevations than the other three clades.

Although *M. kotokui* is composed of clades (putative cryptic species) that are differentiated genetically, ecologically, and morphologically (Ueda *et al.*, 2012; Ueda *et al.*, 2013), the genetic diversity of the clades among mountain regions has not yet been estimated because of a lack of samples from some sites. We hypothesized that genetic diversity of the L3 clade would be lower in the southern mountain regions of Honshu, such as in the Chuo and Minami Alps, around the southern distribution limit of this clade, because global warming might be more likely to lead to fragmentation of habitats and spatial isolation of populations in the southernmost Japan Alps. In this study, we used the highland clade (L3) within *M. kotokui* as an indicator of extinction risk, and compared its genetic diversity among six mountain regions (Myoko, Sugadaira, Kita Alps, Yatsugatake, Chuo Alps, Minami Alps) in the Japan Alps. On the basis of the results, we examined whether global warming has caused ant populations in mountain ecosystems to become isolated.

## Materials and Methods

### Sampling

From June 2009 to September 2012, we haphazardly collected 192 colonies of *Myrmica kotokui* L3 in six mountain regions of Honshu (Myoko, Sugadaira, Kita Alps, Yatsugatake, Chuo Alps, Minami Alps; **Fig. 1**) at elevations ranging from about 1100 to 1900 m a.s.l. (**Table 1**). We used a hand-held



**Fig. 1** Geographical distribution of the highland (L3) clade in *Myrmica kotokui* in the Japan Alps. Dots indicate the collection sites.

GPS unit (eTrex Vista HCx, Garmin, Olathe, KS) to determine the sampling location (latitude, longitude, and elevation). Each sample consisted of 10 workers from a single colony, which were preserved in 99.5% ethanol. Voucher specimens have been deposited at the Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai, Japan.

### Sequence editing and phylogenetic analysis

DNA was extracted from the whole body of a single ethanol-preserved ant from each sample with a DNeasy Blood & Tissue Kit (Qiagen, Hilden) following the manufacturer's protocol. The mitochondrial *cytochrome oxidase I (COI)* gene was amplified by polymerase chain reaction (PCR) by using Takara Ex Taq (Takara Bio, Kusatsu) and primers MyrCOI-F1 (5'-TA GGR TCR CCT GAT ATA GC-3') and MyrCOI-R1 (5'-CC AGG TAY YAT TAA AAT ATA AAC TTC-3') (Ueda *et al.*, 2013), which are at positions 1748 and 2191, respectively, in the *Drosophila yakuba* mtDNA genome. The amplification was carried out for 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 40 s. After amplification, the PCR products were

**Table 1a** Specimens of *Myrmica kotokui* (L3 mtDNA clade) used for the phylogenetic analyses. Dashed lines in the Latitude and Longitude columns indicate missing data. Mountain regions are abbreviated as follows: Chu: Chuo Alps, Kit: Kita Alps, Min: Minami Alps, Myo: Myoko, Sug: Sugadaira, Yat: Yatsugatake. Collectors are abbreviated as follows: SU: Shouhei Ueda, TN & TM: Taito Nozawa and Tetsuya Matsuzuki.

Voucher #	GenBank # COI	Mountain region	Location	Elevation (m a.s.l.)	Latitude	Longitude	Collection Date	Collectors
SU001	LC184029	Kit	Kamikouchi	1573	36°16'44.2"	137°41'249"	2010.10.01	SU
SU003	LC184030	Kit	Kamikouchi	1696	36°16'46.6"	137°41'013"	2010.10.01	SU
SU004	LC184031	Kit	Kamikouchi	1800	36°16'53.9"	137°40'490"	2010.10.01	SU
SU025	LC184032	Kit	Abo	1608	36°12'20.4"	137°36'060"	2011.07.01	SU
SU043	LC184033	Sug	Sugadaira	1237	36°31'10.3"	138°20'16.2"	2011.07.19	SU
SU044	LC184034	Sug	Sugadaira	1237	36°31'09.2"	138°20'16.3"	2011.07.19	SU
SU045	LC184035	Sug	Sugadaira	1142	36°29'36.9"	138°19'10.9"	2011.07.19	SU
SU046	LC184036	Sug	Sugadaira	1421	36°30'10.6"	138°23'27.6"	2011.07.20	SU
SU047	LC184037	Sug	Sugadaira	1594	36°31'09.8"	138°23'54.1"	2011.07.20	SU
SU048	LC184038	Sug	Sugadaira	1594	36°31'09.8"	138°23'54.1"	2011.07.20	SU
SU049	LC184039	Sug	Sugadaira	1594	36°31'09.8"	138°23'54.1"	2011.07.20	SU
SU060	LC184040	Min	Nyukasa	1799	35°53'58.8"	138°10'10.9"	2011.07.29	SU
SU061	LC184041	Min	Nyukasa	1805	35°53'59.6"	138°10'10.2"	2011.07.29	SU
SU062	LC184042	Min	Nyukasa	1797	35°53'59.2"	138°10'10.2"	2011.07.29	SU
SU063	LC184043	Min	Nyukasa	1800	35°53'59.2"	138°10'10.6"	2011.07.29	SU
SU064	LC184044	Min	Nyukasa	1607	35°55'23.0"	138°09'46.5"	2011.07.29	SU
SU066	LC184045	Min	Nyukasa	1605	35°55'21.8"	138°09'45.2"	2011.07.29	SU
SU067	LC184046	Min	Nyukasa	1598	35°55'23.7"	138°09'47.8"	2011.07.29	SU
SU068	LC184047	Min	Nyukasa	1605	35°55'21.9"	138°09'50.0"	2011.07.29	SU
SU069	LC184048	Min	Nyukasa	1330	35°56'46.8"	138°09'31.6"	2011.07.29	SU
SU070	LC184049	Min	Nyukasa	1330	35°56'46.8"	138°09'31.6"	2011.07.29	SU
SU071	LC184050	Min	Nyukasa	1330	35°56'47.3"	138°09'31.9"	2011.07.29	SU
SU073	LC184051	Chu	Nishikoma	1379	35°49'33.0"	137°51'06.1"	2011.08.04	SU
SU075	LC184052	Chu	Nishikoma	1474	35°49'19.2"	137°51'11.7"	2011.08.04	SU
SU076	LC184053	Chu	Nishikoma	1558	35°49'16.0"	137°51'06.8"	2011.08.04	SU
SU077	LC184054	Chu	Nishikoma	1561	35°49'15.8"	137°51'07.1"	2011.08.04	SU
SU078	LC184055	Chu	Nishikoma	1559	35°49'17.0"	137°51'08.5"	2011.08.04	SU
SU079	LC184056	Chu	Nishikoma	1549	35°49'17.2"	137°51'08.9"	2011.08.04	SU
SU080	LC184057	Chu	Nishikoma	1523	35°49'18.0"	137°51'09.8"	2011.08.04	SU
SU081	LC184058	Chu	Nishikoma	1500	35°49'18.3"	137°51'11.0"	2011.08.04	SU
SU083	LC184059	Chu	Nishikoma	1214	35°49'29.5"	137°51'59.0"	2011.08.05	SU
SU084	LC184060	Kit	Kamikouchi	1545	36°15'16.3"	137°40'05.8"	2011.08.10	SU
SU085	LC184061	Kit	Kamikouchi	1647	36°15'25.3"	137°40'03.4"	2011.08.10	SU
SU086	LC184062	Kit	Kamikouchi	1662	36°15'27.0"	137°40'03.3"	2011.08.10	SU
SU087	LC184063	Kit	Kamikouchi	1676	36°15'26.4"	137°40'03.4"	2011.08.10	SU
SU088	LC184064	Kit	Kamikouchi	1712	36°15'30.0"	137°40'00.7"	2011.08.10	SU
SU090	LC184065	Kit	Kamikouchi	1566	36°15'15.6"	137°40'06.1"	2011.08.10	SU
SU091	LC184066	Kit	Kamikouchi	1537	36°15'11.4"	137°40'04.5"	2011.08.10	SU
SU092	LC184067	Kit	Kamikouchi	1537	36°15'11.4"	137°40'04.5"	2011.08.10	SU
SU099	LC184068	Yat	Mugikusa	1901	36°03'06.4"	138°19'09.1"	2011.08.22	SU
SU100	LC184069	Yat	Mugikusa	1901	36°03'06.4"	138°19'09.1"	2011.08.22	SU
SU110	LC184070	Kit	Kamikouchi	1789	36°13'55.6"	137°40'24.1"	2011.08.31	SU
SU114	LC184071	Kit	Kamikouchi	1789	36°13'56.0"	137°40'24.4"	2011.08.31	SU
SU115	LC184072	Kit	Kamikouchi	1789	36°14'10.2"	137°40'20.5"	2011.08.31	SU
SU116	LC184073	Kit	Kamikouchi	1643	36°14'24.6"	137°40'20.5"	2011.08.31	SU
SU117	LC184074	Kit	Kamikouchi	1600	36°14'42.7"	137°40'14.2"	2011.08.31	SU
SU118	LC184075	Kit	Kamikouchi	1636	36°14'42.7"	137°40'14.2"	2011.09.01	SU
SU119	LC184076	Kit	Kamikouchi	1636	36°15'41.2"	137°38'36.2"	2011.09.01	SU
SU122	LC184077	Kit	Kamikouchi	1805	36°15'57.9"	137°38'36.7"	2011.09.01	SU
SU124	LC184078	Kit	Kamikouchi	1780	36°15'55.6"	137°38'35.3"	2011.09.01	SU
SU126	LC184079	Kit	Kamikouchi	1609	36°15'42.3"	137°38'35.9"	2011.09.01	SU
SU127	LC184080	Kit	Kamikouchi	1573	36°15'25.5"	137°38'22.0"	2011.09.01	SU
SU128	LC184081	Kit	Kamikouchi	1755	36°15'34.5"	137°40'00.9"	2011.09.02	SU
SU129	LC184082	Kit	Kamikouchi	1818	36°15'34.9"	137°39'58.5"	2011.09.02	SU
SU130	LC184083	Kit	Kamikouchi	1812	36°15'35.1"	137°39'58.7"	2011.09.02	SU
SU131	LC184084	Kit	Kamikouchi	1795	36°15'34.2"	137°39'59.1"	2011.09.02	SU
SU132	LC184085	Kit	Kamikouchi	1775	36°15'33.5"	137°39'59.5"	2011.09.02	SU
SU134	LC184086	Kit	Kamikouchi	1728	36°15'30.4"	137°40'00.1"	2011.09.02	SU
SU135	LC184087	Kit	Jiigatake	1542	36°33'48.3"	137°43'56.4"	2011.09.08	SU
SU136	LC184088	Kit	Jiigatake	1684	36°33'58.0"	137°44'02.4"	2011.09.08	SU
SU137	LC184089	Kit	Jiigatake	1692	36°33'58.5"	137°44'02.9"	2011.09.08	SU

**Table 1b** (Continued) Specimens of *Myrmica kotokui* used for the phylogenetic analyses.

Voucher #	GenBank # COI	Mountain region	Location	Elevation (m a.s.l.)	Latitude	Longitude	Collection Date	Collectors
SU138	LC184090	Kit	Jiigatake	1780	36°34'06.5"	137°44'04.1"	2011.09.08	SU
SU140	LC184091	Kit	Jiigatake	1824	36°34'09.2"	137°44'08.6"	2011.09.08	SU
SU142	LC184092	Kit	Jiigatake	1875	36°34'12.0"	137°44'11.2"	2011.09.08	SU
SU143	LC184093	Kit	Jiigatake	1875	36°34'12.0"	137°44'11.2"	2011.09.08	SU
SU165	LC184094	Kit	Jiigatake	1911	36°34'14.7"	137°44'12.2"	2011.09.09	SU
SU167	LC184095	Kit	Jiigatake	1713	36°33'56.6"	137°44'00.9"	2011.09.09	SU
SU168	LC184096	Kit	Jiigatake	1686	36°33'55.0"	137°43'58.7"	2011.09.09	SU
SU171	LC184097	Myo	Amakazari	1155	36°53'12.8"	137°58'32.4"	2011.09.29	SU
SU173	LC184098	Myo	Amakazari	1242	36°53'24.0"	137°58'28.9"	2011.09.29	SU
SU174	LC184099	Myo	Amakazari	1344	36°53'30.2"	137°58'25.6"	2011.09.29	SU
SU175	LC184100	Myo	Amakazari	1356	36°53'32.5"	137°58'24.0"	2011.09.29	SU
SU176	LC184101	Myo	Amakazari	1362	36°53'33.1"	137°58'24.1"	2011.09.29	SU
SU177	LC184102	Myo	Amakazari	1388	36°53'36.9"	137°58'21.3"	2011.09.29	SU
SU178	LC184103	Myo	Amakazari	1398	36°53'37.9"	137°58'20.1"	2011.09.29	SU
SU179	LC184104	Myo	Amakazari	1410	36°53'39.6"	137°58'18.4"	2011.09.29	SU
SU180	LC184105	Myo	Amakazari	1484	36°53'49.9"	137°58'19.2"	2011.09.29	SU
SU181	LC184106	Myo	Amakazari	1480	36°53'49.7"	137°58'18.7"	2011.09.29	SU
SU182	LC184107	Myo	Amakazari	1461	36°53'46.3"	137°58'19.4"	2011.09.29	SU
SU183	LC184108	Myo	Amakazari	1436	36°53'43.2"	137°58'18.0"	2011.09.29	SU
SU184	LC184109	Myo	Amakazari	1418	36°53'40.8"	137°58'19.0"	2011.09.29	SU
SU185	LC184110	Myo	Amakazari	1145	36°54'58.4"	137°57'21.6"	2011.10.09	SU
SU195	LC184111	Kit	Kamikouchi	1840	36°15'59.6"	137°38'37.5"	2012.07.03	SU
SU196	LC184112	Kit	Kamikouchi	1826	36°15'58.4"	137°38'37.2"	2012.07.03	SU
SU197	LC184113	Kit	Kamikouchi	1820	36°15'58.2"	137°38'37.1"	2012.07.03	SU
SU199	LC184114	Kit	Kamikouchi	1665	36°15'40.2"	137°38'35.3"	2012.07.03	SU
SU201	LC184115	Kit	Kamikouchi	1750	36°17'40.1"	137°42'10.6"	2012.07.04	SU
SU216	LC184116	Kit	Kamikouchi	1732	36°17'40.7"	137°42'08.9"	2012.07.04	SU
SU217	LC184117	Kit	Kamikouchi	1664	36°17'41.3"	137°42'02.7"	2012.07.04	SU
SU218	LC184118	Kit	Kamikouchi	1567	36°16'38.5"	137°41'36.2"	2012.07.05	SU
SU219	LC184119	Kit	Kamikouchi	1565	36°16'37.6"	137°41'36.3"	2012.07.05	SU
SU220	LC184120	Kit	Kamikouchi	1658	36°17'59.2"	137°41'36.5"	2012.07.05	SU
SU221	LC184121	Kit	Kamikouchi	1672	36°18'05.7"	137°41'30.0"	2012.07.05	SU
SU222	LC184122	Kit	Kamikouchi	1728	36°18'21.3"	137°41'18.3"	2012.07.05	SU
SU223	LC184123	Kit	Kamikouchi	1736	36°18'23.1"	137°41'13.7"	2012.07.05	SU
SU224	LC184124	Kit	Kamikouchi	1773	36°18'27.2"	137°40'56.3"	2012.07.05	SU
SU226	LC184125	Kit	Kamikouchi	1829	36°18'24.5"	137°40'38.4"	2012.07.05	SU
SU232	LC184126	Min	Yashajin	1400	35°38'09.9"	138°20'43.5"	2012.07.27	SU
SU235	LC184127	Min	Yashajin	1509	35°38'14.2"	138°20'33.3"	2012.07.27	SU
SU238	LC184128	Min	Yashajin	1723	35°38'17.5"	138°20'17.5"	2012.07.27	SU
SU240	LC184129	Min	Yashajin	1784	35°38'25.3"	138°20'05.3"	2012.07.27	SU
SU241	LC184130	Min	Yashajin	1776	35°38'28.4"	138°20'03.9"	2012.07.27	SU
SU249	LC184131	Min	Yashajin	1792	35°38'28.4"	138°20'03.4"	2012.07.27	SU
SU250	LC184132	Min	Yashajin	1795	35°38'26.9"	138°20'03.3"	2012.07.27	SU
SU251	LC184133	Min	Yashajin	1789	35°38'16.2"	138°20'11.0"	2012.07.27	SU
SU252	LC184134	Min	Yashajin	1735	35°38'18.7"	138°20'18.6"	2012.07.27	SU
SU253	LC184135	Min	Yashajin	1702	35°38'19.4"	138°20'20.3"	2012.07.27	SU
SU254	LC184136	Min	Yashajin	1588	35°38'19.2"	138°20'29.5"	2012.07.27	SU
SU255	LC184137	Min	Yashajin	1543	35°38'14.6"	138°20'31.8"	2012.07.27	SU
SU256	LC184138	Min	Yashajin	1505	35°38'12.3"	138°20'36.6"	2012.07.27	SU
SU257	LC076940	Kit	Abo	1618	36°12'04.6"	137°35'54.8"	2012.08.30	SU
SU258	LC076941	Kit	Abo	1637	36°12'05.1"	137°35'55.0"	2012.08.30	SU
SU259	LC076942	Kit	Abo	1674	36°12'03.6"	137°35'54.3"	2012.08.30	SU
SU260	LC076943	Kit	Abo	1680	36°12'04.2"	137°35'53.5"	2012.08.30	SU
SU261	LC076944	Kit	Abo	1696	36°12'04.0"	137°35'53.1"	2012.08.30	SU
SU262	LC076945	Kit	Abo	1686	36°12'05.3"	137°35'54.5"	2012.08.30	SU
SU263	LC076946	Kit	Abo	1699	36°12'05.0"	137°35'24.0"	2012.08.30	SU
SU264	LC076947	Kit	Abo	1699	36°12'04.0"	137°35'24.0"	2012.08.30	SU
SU265	LC076948	Kit	Abo	1699	36°12'03.0"	137°35'22.0"	2012.08.30	SU
SU267	LC184139	Sug	Sugadaira	1402	36°31'57.4"	138°21'24.0"	2012.09.06	SU
SU268	LC184140	Sug	Sugadaira	1430	36°31'59.5"	138°21'31.5"	2012.09.06	SU

**Table 1c** (Continued) Specimens of *Myrmica kotokui* used for the phylogenetic analyses.

Voucher #	GenBank # COI	Mountain region	Location	Elevation (m a.s.l.)	Latitude	Longitude	Collection Date	Collectors
SU270	LC184141	Sug	Sugadaira	1543	36°32'13.3"	138°22'07.4"	2012.09.06	SU
SU281	LC184142	Sug	Sugadaira	1801	36°32'34.1"	138°22'55.2"	2012.09.06	SU
SU284	LC184143	Sug	Sugadaira	1862	36°32'36.6"	138°23'01.8"	2012.09.06	SU
SU304	LC184144	Kit	Kamikouchi	1516	36°14'21.2"	137°36'57.0"	2012.09.25	SU
SU305	LC184145	Kit	Kamikouchi	1527	36°14'16.3"	137°36'50.0"	2012.09.25	SU
SU306	LC184146	Kit	Kamikouchi	1530	36°14'15.9"	137°36'49.5"	2012.09.25	SU
SU308	LC184147	Kit	Kamikouchi	1613	36°14'08.5"	137°36'30.4"	2012.09.25	SU
SU309	LC184148	Kit	Kamikouchi	1639	36°14'08.6"	137°36'25.3"	2012.09.25	SU
SU310	LC184149	Kit	Kamikouchi	1671	36°14'07.9"	137°36'17.4"	2012.09.25	SU
SU311	LC184150	Kit	Kamikouchi	1716	36°14'09.3"	137°36'12.5"	2012.09.25	SU
SU312	LC184151	Kit	Kamikouchi	1736	36°14'10.0"	137°36'08.4"	2012.09.25	SU
SU313	LC184152	Kit	Kamikouchi	1814	36°14'07.9"	137°35'56.2"	2012.09.25	SU
SU324	LC184153	Kit	Kamikouchi	1761	36°14'09.0"	137°36'05.6"	2012.09.25	SU
SU325	LC184154	Kit	Kamikouchi	1715	36°14'09.0"	137°36'12.3"	2012.09.25	SU
SU326	LC184155	Kit	Kamikouchi	1583	36°14'08.6"	137°36'36.1"	2012.09.25	SU
SU327	LC184156	Kit	Kamikouchi	1548	36°14'12.6"	137°36'44.7"	2012.09.25	SU
SU329	LC184157	Kit	Kamikouchi	1879	36°16'07.4"	137°38'39.4"	2012.09.26	SU
SU343	LC184158	Kit	Kamikouchi	1538	36°15'11.5"	137°40'06.6"	2012.09.27	SU
SU344	LC184159	Kit	Kamikouchi	1539	36°15'12.4"	137°40'04.7"	2012.09.27	SU
SU345	LC184160	Kit	Kamikouchi	1547	36°15'11.7"	137°40'04.9"	2012.09.27	SU
SU346	LC184161	Kit	Kamikouchi	1548	36°15'12.4"	137°40'04.5"	2012.09.27	SU
SU347	LC184162	Kit	Kamikouchi	1516	36°15'21.9"	137°38'33.2"	2012.09.27	SU
SU348	LC184163	Kit	Kamikouchi	1520	36°15'22.6"	137°38'44.5"	2012.09.27	SU
SU349	LC184164	Kit	Kamikouchi	1522	36°15'16.9"	137°39'01.7"	2012.09.27	SU
SU350	LC184165	Kit	Kamikouchi	1533	36°15'06.6"	137°39'28.8"	2012.09.27	SU
SU351	LC184166	Kit	Kamikouchi	1528	36°15'09.2"	137°39'45.7"	2012.09.27	SU
SU352	LC076949	Kit	Abo	1654	36°12'04.0"	137°35'55.6"	2012.09.28	SU
SU353	LC076950	Kit	Abo	1696	36°12'04.0"	137°35'53.1"	2012.09.28	SU
SU354	LC076951	Kit	Abo	1674	36°12'03.6"	137°35'54.3"	2012.09.28	SU
TN001	AB738420	Chu	Nishikoma	1300	-	-	2009.08.04	TN & TM
TN006	AB738425	Yat	Hara	1260	-	-	2009.08.14	TN & TM
TN008	AB738427	Yat	Sanjiro	1464	36°12'37.8"	138°06'19.5"	2009.09.13	TN & TM
TN009	AB738428	Yat	Tatenoumi	1395	36°04'55.3"	138°08'25.4"	2009.08.04	TN & TM
TN014	AB738432	Sug	Sugadaira	1310	-	-	2009.09.16	TN & TM
TN016	AB738432	Kit	Norikura	1576	36°07'07.4"	137°36'51.6"	2009.09.22	TN & TM
TN020	AB738437	Chu	Ohtaira	1205	35°33'26.8"	137°41'06.3"	2009.09.06	TN & TM
TN025	AB738442	Kit	Norikura	1455	36°07'20.8"	137°37'41.9"	2009.07.23	TN & TM
TN026	AB738443	Kit	Norikura	1473	36°07'20.9"	137°37'42.2"	2009.07.23	TN & TM
TN027	AB738444	Kit	Norikura	1478	36°07'20.5"	137°37'41.8"	2009.07.23	TN & TM
TN032	AB738447	Kit	Sakai	1418	36°02'05.6"	137°42'37.1"	2009.07.24	TN & TM
TN033	AB738448	Kit	Sakai	1408	36°02'06.0"	137°42'37.8"	2009.07.24	TN & TM
TN034	AB738449	Yat	Tateshina	1371	36°03'14.0"	138°16'14.1"	2009.08.04	TN & TM
TN037	AB738452	Kit	Nomugi	1278	36°02'28.2"	137°38'17.3"	2009.08.31	TN & TM
TN038	AB738453	Kit	Nakabusa	1187	36°22'27.6"	137°45'29.1"	2009.09.01	TN & TM
TN039	AB738454	Kit	Nakabusa	1216	36°22'27.3"	137°45'30.6"	2009.09.01	TN & TM
TN044	AB738459	Sug	Sugadaira	1231	36°33'00.8"	138°20'02.8"	2009.09.03	TN & TM
TN045	AB738460	Sug	Sugadaira	1119	36°29'49.5"	138°21'10.3"	2009.09.03	TN & TM
TN046	AB738461	Sug	Shikasawa	1433	36°27'07.1"	138°25'40.2"	2009.09.03	TN & TM
TN047	AB738462	Min	Kurokawa	1686	35°37'50.1"	138°05'36.7"	2009.10.16	TN & TM
TN048	AB738463	Min	Kurokawa	1725	35°37'49.4"	138°05'36.7"	2009.10.16	TN & TM
TN049	AB738464	Kit	Kamikouchi	1580	36°15'46.1"	137°41'47.8"	2009.08.26	TN & TM
TN051	AB738466	Kit	Kamikouchi	1578	36°15'53.5"	137°41'30.5"	2009.08.26	TN & TM
TN052	AB738467	Kit	Kamikouchi	1588	36°15'46.1"	137°41'47.8"	2009.08.26	TN & TM
TN057	AB738472	Kit	Abo	1654	36°12'01.0"	137°35'59.8"	2009.06.28	TN & TM
TN058	AB738473	Kit	Abo	1663	36°12'00.4"	137°35'59.0"	2009.06.28	TN & TM
TN059	AB738474	Kit	Abo	1674	36°12'00.6"	137°35'58.6"	2009.06.28	TN & TM
TN062	AB738475	Sug	Sugadaira	1310	-	-	2009.09.16	TN & TM
TN063	AB738476	Kit	Kamikouchi	1594	36°16'43.1"	137°41'34.6"	2009.09.26	TN & TM
TN064	AB738477	Kit	Kamikouchi	1598	36°16'46.0"	137°41'33.9"	2009.09.26	TN & TM
TN073	AB738478	Sug	Sugadaira	1350	36°30'56.8"	138°21'24.5"	2009.09.16	TN & TM
TN075	LC184167	Kit	Nomugi	1462	36°02'52.7"	137°42'41.6"	2009.09.22	TN & TM
TN090	AB738485	Kit	Kamikouchi	1615	36°15'47.7"	137°41'52.8"	2009.08.26	TN & TM
TN092	AB738487	Min	Kurokawa	1507	35°38'07.6"	138°04'41.4"	2009.09.10	TN & TM
TN093	AB738488	Min	Karei	1768	35°47'56.9"	138°07'21.2"	2009.09.10	TN & TM
TN094	AB738489	Min	Karei	1767	35°47'56.4"	138°07'21.1"	2009.09.10	TN & TM

purified with ExoSap-IT (USB, Cleveland, Ohio). Sequencing of both strands was performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Weiterstadt) on an ABI 3130 Genetic Analyzer.

*COI* sequences were edited and aligned with the SeqScape version 2.5 program (ABI, Weiterstadt). The best-fit substitution model was selected by using Akaike and Bayesian information criteria in the MEGA version 5.2 software package (Tamura *et al.*, 2011). The Tamura 3-parameter model (Tamura, 1992) was selected by both criteria. A network tree was constructed by conducting a maximum likelihood analysis with the Tamura 3-parameter model by using the MEGA version 5.2 software package. Clade support was assessed by 1000 bootstrap replications in the MEGA version 5.2 software package.

### Estimation of genetic diversity

We used Tajima's neutrality test in the Arlequin version 2.0 software package (Schneider *et al.*, 2000) to confirm that all mutations of the *COI* sequences were selectively neutral (Tajima's  $D = 0.16$ ,  $P > 0.05$ ). Then, to test the geographical differentiation of *COI* sequences among the six mountain regions, we conducted an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) with Arlequin ver. 2.0. The results revealed significant differentiation among mountain regions with fixation index  $\Phi_{ST} = 0.45$  ( $P < 0.001$ ) (d.f. = 5, sum of squares = 52.89, variance component = 0.28, percentage of variation = 3.9). On the basis of the results of these two tests, we used all sequence data for estimation of genetic diversity.

To estimate the genetic diversity in each mountain region, we calculated haplotype diversity ( $h$ ) (Nei, 1987) and nucleotide diversity ( $\pi$ ) (Tajima, 1983) with Arlequin ver. 2.0. Haplotype diversity is the probability that two randomly selected haplotypes are different, and nucleotide diversity is the mean proportion of nucleotide sites that differ in all pairwise comparisons. Grant and Bowen (1998) proposed that high  $h$  and high  $\pi$  values indicate a large stable population with a long evolutionary history, high  $h$  and low  $\pi$  indicate a historical bottleneck followed by rapid population growth, low  $h$  and high  $\pi$  indicate divergence between geographically subdivided populations, and low  $h$  and low  $\pi$  indicate a recent bottleneck or a founder event by a single or few haplotypes. If habitat fragmentation and population isolation have occurred in a mountain region as a consequence of global warming, both  $h$  and  $\pi$  should be low in that region.

### Demographic expansion

To test demographic expansion age in each mountain region, we conducted mismatch distribution analysis (Slatkin and Hudson, 1991) with Arlequin ver. 2.0. The analysis estimated the distribution of the observed number of pairwise differences between haplotypes, from which parameters of a sudden demographic expansion model (Schneider and Excoffier, 1999). We calculated the expansion age parameter  $\tau$ , the population size parameters before and after expansion,  $\theta_0$  and  $\theta_1$ . The fit of the expansion model was assessed by the sum of square deviation (SSD) between the observed and the expected mismatch distributions, and significance of the deviations of the observed from the expected distribution was assessed with 1000 replicates of parametric bootstrapping. The expansion age  $t$  was calculated by the expansion age parameter  $\tau$  ( $=2\mu t$ ), where  $\mu$  is the aggregate mutation rate ( $=1.5\%$  divergence per million years; Quek *et al.*, 2004) across all nucleotides per generation (set at 8 years; Quek *et al.*, 2007).

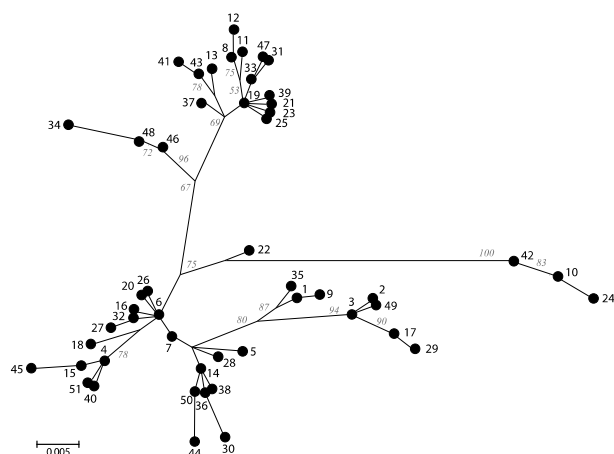
## Results

### Phylogenetic network of *COI*

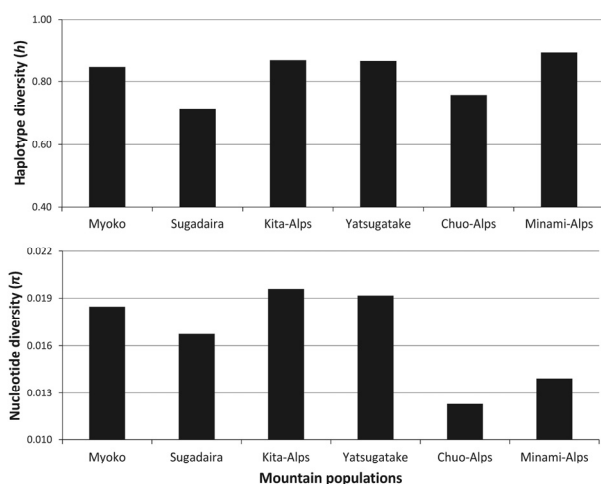
From the 192 L3 clade samples of *Myrmica kotokui* from the six mountain regions (Myoko, Sugadaira, Kita Alps, Yatsugatake, Chuo Alps, Minami Alps, **Fig. 1**), we obtained a mitochondrial *COI* gene dataset consisting of a 455 bp nucleotide sequence. The dataset included 49 bp of synonymous mutations and 3 bp of nonsynonymous mutations. The phylogenetic network analysis of the dataset identified 51 haplotypes (mean genetic distance separating the samples =  $0.024 \pm 0.004$ ; **Fig. 2**).

### Genetic diversity

Using the obtained phylogenetic network, we calculated haplotype diversity  $h$  and nucleotide diversity  $\pi$  and compared their values among the six mountain regions. We could decide that  $h$  values were high ( $h \geq 0.71$ ) in all six mountain regions (Myoko, Sugadaira, Kita Alps, Yatsugatake, Chuo Alps, Minami Alps, **Fig. 3**), because the values were continuous and there were not break among them, and because more than 0.70 value is generally considered high in some previous studies (Grant and Bowen, 1998). Haplotype diversity was highest ( $h = 0.89$ ) in the Minami Alps and lowest ( $h = 0.71$ ) in Sugadaira (**Fig. 3**). Whereas, the  $\pi$  values fell into two clusters with a break observed between 0.014 and 0.017. Thus, we divided the  $\pi$  values into low- $\pi$  ( $\pi \leq 0.014$ ) and high- $\pi$  ( $\pi \geq 0.017$ ) groups (**Fig. 3**). The  $\pi$  values were high in four mountain regions



**Fig. 2** Network phylogeny of the highland (L3) clade of *Myrmica kotokui* based on the DNA sequences of the mitochondrial *COI* gene based on the neighbor-joining method. The length of each branch indicates evolutionary distances based on the Tamura 3-parameter model. The analysis revealed 51 haplotypes (black dots and numbers), and the black dots indicate terminal nodes. Gray numbers above the branches show the bootstrap support.



**Fig. 3** Comparisons of haplotype and nucleotide diversity among the six mountain populations of *Myrmica kotokui*.

(Myoko, Sugadaira, Kita Alps, Yatsugatake) and low in two mountain regions (Chuo Alps, Minami Alps). Nucleotide diversity was highest ( $\pi = 0.020$ ) in the Kita Alps and lowest ( $\pi = 0.012$ ) in the Chuo Alps.

#### Demographic history

The mismatch distributions on Kita-Alps, Yatsugatake, Chuo-Alps and Minami-Alps populations were not significantly different from the expected expansion model under the SSD test, and the expansion ages of these

populations were estimated to 1.01, 0.62, 0.91, 0.82 million years ago (Ma), respectively (**Table 2**). We did not infer the expansion ages in Myoko and Sugadaira populations, because their mismatch distributions were significantly different from the expansion model (**Table 2**).

#### Discussion

Both  $h$  and  $\pi$  were high in four mountain regions (Myoko, Sugadaira, Kita Alps, Yatsugatake, **Fig. 3**), suggesting large stable populations with a long evolutionary history (Grant and Bowen, 1998). The high haplotype and nucleotide diversity in these four regions of the Japan Alps may indicate that they served as interglacial refugia for mountain animals. In contrast, in the southernmost regions, the Chuo and Minami Alps,  $h$  was high and  $\pi$  was low (**Fig. 3**), suggesting that a historical bottleneck was followed by rapid population growth (Grant and Bowen, 1998). And we inferred the bottleneck event occurred by a few million years ago, because the mismatch distribution analysis indicated that the expansion ages of the Chuo and Minami Alps populations were estimated at 0.91 and 0.82 million years ago, respectively (**Table 2**). This result suggests that the low nucleotide diversity in the southernmost mountain regions was not a consequence of global warming but of some geological event that occurred, perhaps, a few million years ago. The genetic diversity pattern in the southernmost mountain regions indicates that in these populations there were many haplotypes, but no rare haplotypes that were genetically distant from the major haplotypes. In fact, the four genetically most distant haplotypes (numbers 10, 22, 24, 42; **Fig. 2**) did not occur in the Chuo and Minami Alps. Owing to their low frequency, these haplotypes might have become extinct by chance as a result of a severe population decline during the last inter-glacial period.

We conclude from our population genetic analysis of ants inhabiting medium altitude sites in the Japan Alps that these mountain ecosystems in the Japan Alps may not presently face a crisis of extinction from habitat fragmentation and population isolation resulting from global warming. In other words, the results of this population genetic analysis may indicate an early stage of genetic diversity in these ecosystems just before global warming began to have an impact. Continuing research on the dynamics of genetic diversity in mountain ants will thus provide basic quantitative information on the impacts of global warming on these mountain ecosystems.

In the Japan Alps, two other ant species, *M. kurokii* and *Formica gagatoides*, are distributed in alpine areas that are

**Table 2** Parameters of mismatch distribution analysis, the expansion age parameter ( $\tau$ ), the population size parameters before expansion ( $\theta_0$ ) and after expansion ( $\theta_1$ ), the expansion age ( $t$ ).

Mountain region	Mismatch Distribution			SSD	$P$ (SSD)	Expansion Age $t$ (Ma)
	$\tau$	$\theta_0$	$\theta_1$			
Myoko	11.40	0.01	15.68	0.07	*	-
Sugadaira	9.50	0.00	7.63	0.11	*	-
Kita-Alps	12.11	0.00	13.45	0.03	n.s.	1.01
Yatsugatake	7.40	3.84	28.36	0.10	n.s.	0.62
Chuo-Alps	10.96	0.00	7.83	0.07	n.s.	0.91
Minami-Alps	9.89	0.00	11.32	0.02	n.s.	0.82

expected to be strongly affected by global climate changes (Nakamura, 2007). Further population genetic analyses of these other two alpine ant species should clarify the degree to which local extinction has occurred in alpine ecosystem in Japan.

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## シワクシケアリ高地系統の集団遺伝構造解析

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日本の山岳生態系は地球規模の温暖化の影響を受け、消失の危機にさらされているとされる。我々は、中部山岳の生態系が地球温暖化の影響を受けて分断化・孤立化を起こしているか否かを、シワクシケアリ高地系統を用いた集団遺伝学的解析から明らかにすることを目的とした。中部山岳地域の6山域から採取したシワクシケアリ高地系統192サンプルを用いて、ミトコンドリアDNAのチトクロームオキシダーゼI遺伝子(COI)の部分配列に基づく分子系統樹を作成した。次に、得られた分子系統樹に基づき、遺伝的多様度(ハプロタイプ多様度と塩基多様度)を山域ごとに算出し、比較した。その結果、ハプロタイプ多様度は全ての山域で高い水準を維持しているが、塩基多様度はより南方の山域(中央アルプスと南アルプス)で低下していることが明らかになった。より南方の山域におけるハプロタイプ多様度は高く、塩基多様度は低いというパターンは、数百万年前に集団がボトルネック効果を受け、小集団化を経験した後に、集団サイズが成長したことを示している。つまり、より南方の山域でみられた塩基多様度の低下は、近年の温暖化の影響を受けていないと考えられる。