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Genetic structure and phylogeography of a temperate-boreal herb, *Cardamine scutata* (Brassicaceae), in northeast Asia inferred from AFLPs and cpDNA haplotypes[1]

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- *Premise of the study:* Studies on genetic structure of plant populations help us understand the history of local flora and vegetation. In this study, we focus on the temperate-boreal herb *Cardamine scutata* from northeast Asia, an area with scarce phylogeographic studies. We explore patterns of genetic variation within this species, with an aim to infer its (post-) glacial history with reference to colonization routes and migrations via land bridges.
- *Methods:* We analyzed 46 populations sampled in Japan, Kamchatka, and Korea using AFLP and cpDNA sequence data.
- *Key results:* Two intraspecific genetic groups were resolved, distributed in the northeastern and southwestern part of the study area, most likely reflecting lineages isolated from each other during (at least) the last glaciation. A zone of secondary contacts was found in central/northern Honshu, and a few cases of long-distance dispersal were observed. We detected efficient gene flow across the marine straits, supporting the role of land bridges created by sea level decline during the last glacial period. The cpDNA data indicated extensive recent expansion and diversification within both lineages. We inferred recent colonization of Kamchatka from Hokkaido, associated with genetic impoverishment.
- *Conclusions:* The pattern of north–south genetic differentiation found in *C. scutata* is rather common among several other plant species studied in Japan, despite their distinct biological features. We assume that different processes and factors may have brought about this similarity. Overall, this study contributes to better understanding of the biogeography of northeast Asia.

Key words: AFLP; Brassicaceae; *Cardamine*; cpDNA haplotypes; Japan; Kamchatka; phylogeography.

Patterns of genetic diversity within widespread plant species are shaped by the interaction of many factors, such as life history traits and ecological variables (e.g., life cycle, breeding system, pollination and dispersal mechanisms, effective population size and connectivity, and human impact; Loveless and Hamrick, 1984; Eckert et al., 2008; Palstra and Ruzzante, 2008), but also historical events. The effects of ice ages on temperate flora were considerable, involving latitudinal and altitudinal migrations, population extinction, and range fragmentation and/or expansion (Hewitt, 2000). Molecular markers have become a powerful tool for inferring recent and past demographic processes, and the (post-) glacial histories of plant species (e.g., Eidesen et al., 2007; Beck et al., 2008; Maki et al., 2008).

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The Japanese archipelago, which consists of four main islands extending over 2000 km in the southwest–northeast direction, encompasses a wide range of climatic zones and many floral and vegetation types from warm-temperate, evergreen broadleaf forests to temperate, deciduous broadleaf and subboreal coniferous forests. Environmental conditions are strikingly different also between the Japan Sea side and the Pacific side of the archipelago (Yoshino, 1980). During the last glacial period, there were no major glaciers in Japan, but both temperature and precipitation were significantly lower than today (Tsukada, 1983). Reconstructions of past vegetation indicate that the main vegetation zones were displaced toward the south and lower altitudes (see Dobson 1994: fig. 4). Boreal coniferous forests, now present on mountain tops of northern Honshu and in northern Hokkaido, dominated across Honshu and southern Hokkaido, with a more open tundra environment spreading in northern Hokkaido. Temperate, deciduous forests, now prevalent across central and northern Honshu, were pushed to coastal regions of central Honshu, to southern Honshu, Shikoku, and Kyushu, while evergreen broadleaf forests were limited to the Pacific coast of the southernmost areas (Tsukada, 1983; reviewed by Dobson, 1994 and Millien-Parra and Jaeger, 1999). Glaciers, however, were locally present in the central and northern Kuril Islands, in Kamchatka, and also partly covered the Okhotsk Sea (Frenzel et al., 1992; Pietsch et al., 2003). Importantly, the large volume of accumulated ice caused sea-level regressions, reducing the depth and width of marine straits and creating several land-bridge connections. The connections occurred between Shikoku, Kyushu, and Honshu in the south and

between Hokkaido, the southern Kurils, and the Asian continent via Sakhalin in the north. The Korean Peninsula remained probably separated from Honshu and Kyushu, but the strait was significantly reduced (Japan Association for Quaternary Research, 1977; Ohshima, 1990; Pietsch et al., 2003). Fluctuations in sea level may have affected the availability and connectivity of appropriate habitats and facilitated oversea plant migrations and changes in distributions.

Several molecular studies have been undertaken in recent years with the major goal of understanding the Quaternary history of plant species in eastern Asia. Most of these studies have concentrated on either alpine plants (e.g., Fujii and Senni, 2006; Ikeda et al., 2008a, b) or trees and shrubs (e.g., Tsuda and Ide, 2005; Okaura et al., 2007; Aizawa et al., 2009) within the area of Japan. Only a few phylogeographical studies have focused on lower-altitude temperate herbs, either considering the subtropical, warm-temperate region of south Japan, eastern China, and Korea (Li et al., 2008; Maki et al., 2008; Qiu et al., 2009), or the temperate zone across larger latitudinal ranges (Inamura et al., 2000; Koga et al., 2008). The responses of such plants to Pleistocene glaciations, including range shifts, migration routes, dispersal events across land bridges, and population divergence, are still poorly understood.

Cardamine scutata Thunb. (Brassicaceae) is a common herb distributed in northeast Asia. Based on data from literature (Berkutenko, 1988; Lee, 1996; Zhou et al., 2001; Al-Shehbaz et al., 2006), herbaria (LE and VLA in Russia, PE in China, and numerous herbaria in Japan), and field surveys (H. Kudoh, K. Marhold, unpublished data), the center of its distribution is the Japanese Archipelago, Kurils, Sakhalin, and Kamchatka (south of 45°N). Rarely and often only temporarily, it is also found in coastal regions of Magadanskaya Oblast', Primorskii and Khabarovskii Krai of the Russian Far East, in the coastal province of Zhejiang of China, and in the central part of the Korean Peninsula. Records from inland provinces of China are dubious. The species is tetraploid (Lihová et al., 2006), apparently of an allopolyploid origin (K. Shimizu, University of Zürich, Switzerland, unpublished data), perennial, highly inbreeding (Kimata, 1983; H. Kudoh, personal observation), and capable of vegetative spread by regrowing from stem segments (H. Kudoh, personal observation). It grows in wet to muddy sites on field and road margins, in wet depressions, and along creeks from sea level up to 1600 m. The seeds lack any specific adaptations for long-distance dispersal; they are dispersed by the winding elasticity of the assumenta of a silique, falling close (ca 1.5 m) to the maternal plant, but they are small and light, produced in high quantities (1300–3100 per plant), and thus easily dispersed by wind and flowing water (Kimata, 1983) and probably by migrating birds (Watanabe, 2008) to longer distances. Species' broad altitudinal and latitudinal range implies substantial thermal tolerance, which may have enhanced survival during the Late Pleistocene, although it requires wet habitats.

In this study, we explored patterns of genetic diversity in *C. scutata* in northeast Asia using amplified fragment length polymorphisms (AFLPs; Vos et al., 1995) and cpDNA sequences. We were interested in inferring the population history of this species and assessing the impact of Pleistocene glaciation. The efficiency and merits of cpDNA markers, which are uniparentally inherited, nonrecombinant, relatively slowly evolving, have been demonstrated in previous studies tracing glacial refugia, migration routes, and range dynamics (e.g., Beck et al., 2008; Ronikier et al., 2008). AFLP, a high-resolution genotyping technique, screens many (mainly nuclear) loci and yields highly polymorphic data that are used to address a number of phylogeographical questions (e.g., Schönswetter et al., 2006; Ehrich et al., 2008).

MATERIALS AND METHODS

Plant material, DNA extraction, and marker selection—We sampled 46 populations of *Cardamine scutata* across the Japanese Archipelago, in Kamchatka, and in South Korea (Table 1, Fig. 1, Appendix 1), thus, covering a significant part of the species distribution (see introduction). Due to logistic reasons, we were not able to acquire material from Sakhalin. In addition, a few populations morphologically attributable to its close relative, a local Japanese endemic *C. longifructus* Ohwi, were included (see Table 1). We sampled the studied populations mostly along streams and collected the samples from distant individuals (>5–10 m apart; occasionally in small populations the distance was lower, but always >2 m) to avoid sampling from a clone as much as possible. Fresh leaf samples were collected and dried in silica gel. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

For AFLPs, we typically used 5–7 (rarely 4 or 8) plants per population, totaling 306 individuals (Table 1). Because *C. scutata* is a widespread and highly selfing species, we have chosen a sampling strategy that aims to analyze as many populations as possible, although at the cost of lower sample size. This strategy should be efficient in revealing the overall variation patterns and the main trends (see Schönswetter et al., 2008; Ortiz et al., 2008; Huck et al., 2009; Skrede et al., 2009), although some intrapopulation estimates may be inaccurate, limiting interpretations of the obtained data in some cases.

We also surveyed polymorphisms in several cpDNA regions and finally selected two intergenic spacers, *trnL*$^{(UAA)}$-*trnF*$^{(GAA)}$ and *rpl32*-*trnL*$^{(UAG)}$ (Shaw et al., 2007). The former has been commonly used in Brassicaceae (e.g., Dobeš et al., 2004; Beck et al., 2008), including *Cardamine* (e.g., Lihová et al., 2006). Part of its 3′ region, which is affected by the pseudogenization of the *trnF* gene (see Koch et al., 2005), was eliminated from analyses to avoid ambiguity in sequence alignment and indel handling. Since *trnL-trnF* sequences were already available for several *Cardamine* taxa (19 taxa, including *C. scutata*, sampled worldwide in Lihová et al., 2006), they allowed rooting the here generated haplotype network of *C. scutata* with taxa from sister clades (*C. pensylvanica*, *C. parviflora*), and identifying ancestral haplotypes. The *rpl32-trnL*$^{(UAG)}$ region has not been employed so far in Brassicaceae, but was chosen because our initial surveys indicated greater polymorphisms than in other regions. Both cpDNA regions were sequenced in 3–4 plants per population, altogether in 172 individuals (Table 1).

AFLP fingerprinting—AFLP analysis followed the general procedure described by Vos et al. (1995) and the protocol provided by Applied Biosystems (2005), with some modifications as given in Mereďa et al. (2008). Selective primer pairs were initially screened on a few samples from four populations. Of the 17 primer pair combinations tested, four pairs were selected that gave the best results with respect to polymorphism and clarity of AFLP profiles: *Eco*RI-ATC-(6-FAM)/*Mse*I-CAG, *Eco*RI-AAG-(VIC)/*Mse*I-CTG, *Eco*RI-AAC-(NED)/*Mse*I-CAT, and *Eco*RI-AAG-(PET)/*Mse*I-CAC. Fragment analysis was performed on an ABI 3100-Avant sequencer (Applied Biosystems, Foster City, California, USA) at the BITCET Consortium, Comenius University, Bratislava. For size calibration, we used the internal size standard GeneScan –500 LIZ (Applied Biosystems).

AFLP data analysis—Raw AFLP data were collected and scored using the programs GeneScan 3.7 (Applied Biosystems) and Genographer 1.6.0 (Montana State University, available at website http://hordeum.msu.montana.edu/genographer/). Unambiguously scorable fragments in the size range 75–500 bp were recorded and coded as present (1) or absent (0). To estimate the reproducibility of the AFLP data (Bonin et al., 2004), DNA from 15 samples (5% of the final data set) was extracted twice, and the replicated samples were analyzed and scored independently.

The relationships among populations and individuals were first explored by principal coordinate analysis (PCoA) and by constructing a neighbor-joining (NJ) tree. PCoA was performed by the software famd 1.108 beta (Schlüter and Harris, 2006) using Jaccard's similarity coefficient. The NJ tree, based on Nei and Li's (1979) genetic distance, was constructed in PAUP* version 4.0b10 (Swofford, 2001); group support was assessed by a bootstrap analysis with 5000 replicates. The tree was rooted by the populations attributable to *C. longifructus* that appeared as genetically distict (see Results). Bayesian model-based

TABLE 1. List of 46 populations of *Cardamine scutata* and their genetic characteristics. Populations of the related *C. longifructus*, distinct in AFLP variation but placed within the overall cpDNA variation, are listed at the end of the table with a superscript "a".

				AFLP					
Pop. code	Country, region	Locality	Latitude (N)/ longitude (E)	*n*/*n* geno	*n*/ind	*P* (%)	D_{Nei}	DW1	cpDNA
Kor	S Korea	Mt. Odaesan, Gangwon	37.74/128.59	7/1	62 ± 0	0	0	0.4452	S1,S1,S1
Kyu1	JP, Kyushu	Sagara, Kumamoto	32.31/130.84	6/4	66 ± 2	7.21	0.0254	0.9175	S2,S2,S2
Kyu2	JP, Kyushu	Itsuki, Kumamoto	32.45/130.79	6/2	67 ± 0	0.33	0.0011	0.7183	S2,S2,S2
Kyu3	JP, Kyushu	Misato, Kumamoto	32.58/130.89	6/5	66 ± 2	2.62	0.0109	0.5244	S7,S7,S7
Kyu4	JP, Kyushu	Mt. Shiraiwa, Miyazaki	32.56/131.15	5/3	76 ± 14	13.77	0.0826	3.4494	S11,*,*
Shi1	JP, Shikoku	Omogo, Ehime	33.75/133.00	6/1	69 ± 0	0	0	1.2462	S9,S9,S9,S9
Shi2	JP, Shikoku	Matsuyama, Ehime	33.79/132.81	4/4	67 ± 2	5.90	0.0350	1.8470	S1,S1,S1,S20
Shi3	JP, Shikoku	Seiyo, Ehime	33.49/132.82	5/5	64 ± 2	3.61	0.0200	0.8243	S16,S16,S16,S16
Shi4	JP, Shikoku	Seiyo, Ehime	33.44/132.72	6/1	63 ± 0	0	0	0.4335	S16,S16,S16,S16
SHo1	JP, Honshu	Akiohta, Hiroshima	34.56/132.28	6/6	67 ± 4	7.21	0.0337	0.8239	S2,S2,S2
SHo3	JP, Honshu	Tatsuno, Hyogo	34.99/134.50	6/6	71 ± 9	17.71	0.0689	1.9276	S1,S1,S1,*
SHo4	JP, Honshu	Haga, Hyogo	35.21/134.49	6/5	64 ± 1	2.62	0.0118	0.4864	S3,S3,S3
SHo6	JP, Honshu	Asago, Hyogo	35.41/134.92	6/6	69 ± 6	9.51	0.0413	0.7379	S1,S1,S16,S16
SHo8	JP, Honshu	Taka, Hyogo	35.08/134.93	8/3	67 ± 0	0.66	0.0022	0.4716	S1,S1,S1,S1
CHo1	JP, Honshu	Gujyo, Gifu	35.77/137.03	6/6	68 ± 4	6.89	0.0315	0.6858	S5,S5,S6
CHo2	JP, Honshu	Kiso, Nagano	35.99/137.59	6/5	72 ± 2	8.85	0.0442	0.9583	S1,S1,S1
CHo3	JP, Honshu	Kamikochi, Nagano	36.26/137.68	6/2	74 ± 0	0.33	0.0011	0.8099	S18,S18,S18
CHo4	JP, Honshu	Ena, Gifu	35.47/137.37	6/5	68 ± 1	8.52	0.0383	0.6042	S1,S1,S1,S1
CHo5	JP, Honshu	Komagane, Nagano	35.74/137.89	6/5	71 ± 3	5.57	0.0223	1.3304	S12,S12,S12
CHo6	JP, Honshu	Toyama, Toyama	36.48/137.23	6/6	69 ± 2	6.56	0.0323	0.4745	S1,S1,S14
CHo7	JP, Honshu	Takayama, Gunma	37.64/138.96	6/5	69 ± 4	6.23	0.0278	0.8450	S4,S4,S10
CHo8	JP, Honshu	Sanada, Nagano	36.48/138.35	6/4	72 ± 5	8.52	0.0385	0.5397	S1,S1,N10
CHo9	JP, Honshu	Nikko, Tochigi	36.75/139.42	7/3	67 ± 2	4.26	0.0215	0.5233	N3,N3,S19
CHo10	JP, Honshu	Nikko, Tochigi	36.76/139.41	6/2	70 ± 1	2.30	0.0077	0.8390	S19,S19,S19
CHo11	JP, Honshu	Yunotani, Niigata	37.12/139.16	6/2	64 ± 0	0.33	0.0011	0.4230	S1,S1,S1
NHo1	JP, Honshu	Kanagi, Aomori	40.90/140.53	6/5	68 ± 4	8.20	0.0376	0.6370	N9,N9,N1
NHo2	JP, Honshu	Iwaizumi, Iwate	39.71/141.45	7/5	72 ± 2	6.89	0.0231	0.7445	N8,N8,N8
NHo3	JP, Honshu	Kuzumaki, Iwate	40.01/141.54	5/3	65 ± 1	7.87	0.0472	0.6535	N11,N12,N12,N12
NHo4	JP, Honshu	Hachimantai, Akita	39.98/140.79	6/3	68 ± 1	9.51	0.0511	0.4458	S1,S1,N1
Hok1	JP, Hokkaido	Bifuka	44.57/142.41	6/1	68 ± 0	0	0	0.8984	N7,N7,N7
Hok2	JP, Hokkaido	Kamiiso	41.81/140.54	5/3	66 ± 2	9.51	0.0413	0.5172	S17,N2,N4
Hok3	JP, Hokkaido	Higashikagura	43.65/142.49	6/3	71 ± 1	0.98	0.0039	0.6662	N13,N13,N13
Hok4	JP, Hokkaido	Otaru	43.09/141.06	6/3	73 ± 1	1.64	0.0090	0.4844	N6,N6,N6
Hok5	JP, Hokkaido	Yakumo	42.18/140.13	4/1	67 ± 0	0	0	0.2329	N9,N9,N9,N9
Hok6	JP, Hokkaido	Setana	42.52/139.91	5/5	70 ± 2	13.44	0.0695	1.0980	S13,S13,N5,N8
Hok7	JP, Hokkaido	Sarufutsu	45.18/142.15	5/5	73 ± 2	4.26	0.0230	0.8193	N14,N14,N14
Hok8	JP, Hokkaido	Urakawa	42.24/142.93	5/3	69 ± 1	1.97	0.0111	0.2838	S1,S1,S1
Hok9	JP, Hokkaido	Kamishihoro	43.32/143.04	6/6	71 ± 5	7.54	0.0297	1.3144	N1,N1,N1,N1
Hok10	JP, Hokkaido	Honbetsu	43.16/143.68	6/5	72 ± 2	5.90	0.0275	0.9026	N13,N13,N14
Kam1	Ru, Kamchatka	Elizovo	53.21/158.53	6/3	72 ± 1	0.66	0.0031	0.3332	N1,N1,N1
Kam2	Ru, Kamchatka	Esso	55.90/158.69	6/5	74 ± 1	1.31	0.0070	0.3438	N1,N1,N1
Kam3	Ru, Kamchatka	Esso	55.92/158.72	6/5	73 ± 2	1.97	0.0087	0.3329	N1,N1,N1
Kam4	Ru, Kamchatka	Nachikinskoe ozero	53.49/157.78	6/3	71 ± 0	0.98	0.0050	0.4940	N1,N1,N1
Kam5	Ru, Kamchatka	Pushchino	54.02/157.85	6/4	73 ± 1	1.64	0.0068	0.3817	N1,N1,N10
Kam6	Ru, Kamchatka	Nachiki	53.12/157.75	6/1	71 ± 0	0	0	0.3556	N1,N1,N1
Kam7	Ru, Kamchatka	Pushchino	54.27/158.12	5/4	72 ± 1	7.21	0.0302	0.5875	N1,N1,N10,N10
SHo2 [a]	JP, Honshu	Tatsuno, Hyogo	34.99/134.50	6/1	—	—	—	—	S1,S1,S1
SHo5 [a]	JP, Honshu	Haga, Hyogo	35.21/134.49	6/1	—	—	—	—	S15,S15,S15
SHo7 [a]	JP, Honshu	Taka, Hyogo	35.08/134.89	6/2	—	—	—	—	S1,S1,S1,S1
SHo9 [a]	JP, Honshu	Sasayama, Hyogo	35.02/135.16	5/2	—	—	—	—	S1,S1,S1
CHo12 [a]	JP, Honshu	Iwaki, Fukushima	37.19/140.93	5/5	—	—	—	—	S15,S15,S15
Shi5 [b]	JP, Shikoku	Mt. Tsurugi, Tokushima	33.86/134.10	5/1	—	—	—	—	S8,S8,S8
Shi6 [b]	JP, Shikoku	Mt. Miune, Tokushima	33.85/133.99	4/3	—	—	—	—	S8,S8,S8

Notes: Population (Pop.) code, country and region (JP, Japan; Ru, Russia), brief locality (full localities including the voucher information are listed in Appendix 1), and geographic coordinates are given for each site. AFLP columns: *n*/*n* geno, number of plants analyzed in AFLP/number of distinct AFLP phenotypes identified; *n*/ind, average number of fragments per individual ± SD; *P* (%), percentage of polymorphic markers; D_{Nei}, Nei's gene diversity; DW, frequency down-weighted marker values. cpDNA column: *trnL-trnF* and *rpl32-trnL* haplotypes (each letter refers to a single individual; asterisks denote more distant haplotypes). Superscript "b" indicates two eastern Shikoku populations that clustered together with the typical *C. longifructus*, but are morphologically distinct, and may represent a new, so far undescribed species.

clustering methods implemented in STRUCTURE 2.2.3 (Falush et al., 2007) and BAPS 3.2 (Corander et al., 2006) were also applied. For the former, which is based on a Markov chain Monte Carlo algorithm, we used a model with admixture and assumed independence of allele frequencies among populations. The number of clusters (*K*) was set from 1 to 10. To test the stability of the results, ten runs were performed for each *K*. The length of the burn-in period was set to 100 000, and the MCMC chains were run for an additional 1 000 000 replicates after burn-in. The STRUCTURE computations were carried out using the freely

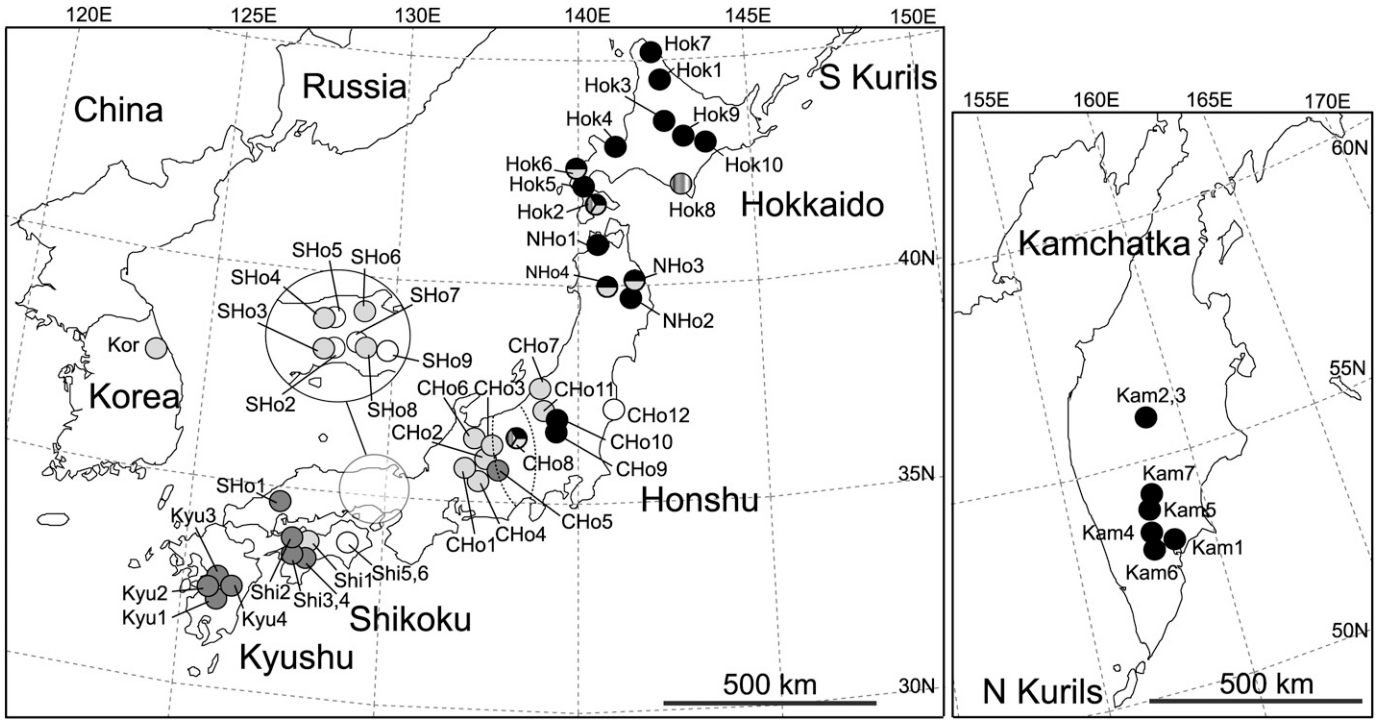


Fig. 1. Map showing sampling sites of *Cardamine scutata*. Genetic groupings based on AFLP data (see Figs. 2, 3) are shown by symbol color: black, northern N group; gray, southern S group (dark and light gray circles indicate two subgroups within S). Shading indicates the presence of genetically intermediate individuals; half- or third-divided circles refer to heterogeneous populations. White symbols indicate a related species *C. longifructus*. Dashed lines drawn in central Honshu show approximate positions of mountain ranges of the Chubu district discussed in the text. Lists of localities and population abbreviations are given in Table 1 and Appendix 1.

accessible Bioportal (University of Oslo, Norway, website http://www.bioportal.uio.no). The R-script STRUCTURE-sum-2009 (part of AFLPdat; Ehrich, 2006) was used to summarize the output files, to calculate similarity coefficients between the replicate runs and to plot the means of the estimated log posterior probability of the data over the replicate runs for each *K* value, denoted as mean *L*(*K*) (see Evanno et al., 2005). Graphical output was generated using CLUMPP ver. 1.1.1 (Jakobsson and Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) software. Another Bayesian clustering analysis, based on stochastic optimization, was performed in BAPS 3.2. It estimates the highest probability partition, i.e., the optimal number of clusters and assignment of the analyzed individuals. Both the frequencies of AFLP markers and the number of genetically divergent groups were treated as random variables. The analysis was repeated five times with the maximum number of clusters (*K*) set to 46 (corresponding to the total number of populations).

Population groupings revealed by the clustering analyses were tested by analyses of molecular variance (AMOVA) based on Euclidean pairwise distances using Arlequin 3.11 (Excoffier et al., 2005). Significance of differentiation among groups was tested using permutation tests as implemented in Arlequin 3.11. Genetic diversity of populations was assessed by calculating the number of distinct AFLP phenotypes, the percentage of polymorphic markers [P(%)], and the average proportion of pairwise differences between individuals (Nei's gene diversity) using the R-script AFLPdat (Ehrich, 2006). As a measure of divergence, we calculated the number of private markers (those restricted to a given population/group), and the frequency down-weighted marker value (Schönswetter and Tribsch, 2005) as implemented in AFLPdat (DW1; Ehrich, 2006).

cpDNA amplification and sequencing—Universal primers were used for both PCR (polymerase chain reaction) and cycle-sequencing: primers e and f for the *trnL*$^{(UAA)}$-*trnF*$^{(GAA)}$ intergenic spacer (Taberlet et al., 1991) and primers rpL32-F and trnL$^{(UAG)}$ for the *rpl32*-*trnL*$^{(UAG)}$ spacer (Shaw et al., 2007). The PCR mix contained 0.75 U of *Pfu* polymerase (Fermentas, St. Leon-Rot, Germany), 1× reaction buffer supplied with the enzyme that included $MgSO_4$ at 2 mM, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1 μL of DNA template in a total reaction volume of 25 μL. Amplifications were run in a Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany). For the *trnL*$^{(UAA)}$-*trnF*$^{(GAA)}$ region, the following temperature profile was used: 94°C (5 min); 35 cycles of 94°C (1 min), 54°C (1 min), and 72°C (45 s); and final extension at 72°C (10 min). For the *rpl32*-*trnL*$^{(UAG)}$ region, we used the "*rpl16*" program given by Shaw et al. (2005). PCR products were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany). Sequencing was performed on an ABI PRISM 3130*xl* sequencer at the BITCET Consortium, Comenius University, Bratislava.

cpDNA data analysis—The cpDNA sequences were edited and aligned manually using the program BIOEDIT (version 7.0.4.1; Hall, 1999). The alignments of both cpDNA regions were combined to a single data set. The method of statistical parsimony was employed to determine the relationships among the haplotypes and to construct the haplotype network (TCS version 1.18; Clement et al., 2000). TCS was run with gaps coded as missing data, and larger indels (see below) coded as additional characters.

Genetic diversity and differentiation were estimated following Pons and Petit (1996) as implemented in the program PERMUT (website http://www.pierroton.inra.fr/genetics/labo/Software/). Both the estimates that take into account the genetic distance (number of pairwise differences; also called estimates based on ordered haplotypes) between haplotypes and those that ignore it (estimates based on unordered haplotypes) were calculated. These included the mean within-population genetic diversity (v_S, h_S), the total genetic diversity (v_T, h_T), and the coefficients of genetic differentiation over the studied populations (N_{ST}, G_{ST}) (for details see Table 2). A permutation approach (10 000 permutations) was used to test whether N_{ST} was significantly greater than G_{ST}, i.e., whether there was a correspondence between haplotype similarities and their geographic distribution. Genetic differentiation among populations and groups was also explored using AMOVA based either on gene diversity (conventional *F*-statistics) or nucleotide diversity (pairwise distances), using Arlequin 3.11 (Excoffier et al., 2005).

RESULTS

AFLPs—In total, 305 AFLP markers were scored in 306 individuals belonging to all 53 populations, and 182 different

TABLE 2. Chloroplast marker [merged *rpl32-trnL*$^{(UAG)}$ and *trnL*$^{(UAA)}$-*trnF*$^{(GAA)}$ intergenic spacers] diversity and differentiation parameters of *Cardamine scutata* estimated for the total data set as well as for the two major cpDNA lineages.

	Number of									
	Pop.	Ind.	Haplotypes	h_S unordered	h_T unordered	G_{ST}	v_S ordered	v_T ordered	N_{ST}	$N_{ST} > G_{ST}$
North	17	55	14	0.186 (0.0727)	0.750 (0.0974)	0.751 (0.0948)	0.145 (0.0600)	0.751 (0.1480)	0.807 (0.0785)	no
South	30	97	20	0.106 (0.0441)	0.832 (0.0592)	0.873 (0.0534)	0.081 (0.0352)	0.833 (0.1242)	0.903 (0.0452)	no
Total	47	152	34	0.135 (0.0385)	0.901 (0.0272)	**0.850 (0.0425)**	0.049 (0.0151)	0.902 (0.0687)	**0.945 (0.0162)**	**yes, $P < 0.01$**

Notes: h_S, v_S indicate mean within-population gene diversity based on unordered and ordered haplotypes, respectively; h_T, v_T are total gene diversity based on unordered and ordered haplotypes, respectively; N_{ST}, G_{ST} are the coefficients of genetic differentiation taking into account the genetic distance between haplotypes or ignoring it, respectively. Standard errors are given in parentheses. $N_{ST} > G_{ST}$: permutation test (10 000 permutations) for the presence of phylogeographic structure. Heterogeneous populations affected by secondary contacts are omitted here (Kyu4, NHo4, CHo8, CHo9, Hok2, Hok6).

multilocus AFLP phenotypes were resolved. Control replicates indicated high reproducibility of the AFLP data (> 99%). When considering only *C. scutata* (i.e., excluding *C. longifructus*), 253 AFLP markers were scored in 268 individuals (46 populations), with 27 markers (10.7%) being monomorphic. We detected 166 different multilocus AFLP phenotypes; identical phenotypes were found within several populations, but also between some Kamchatkan populations, and between two populations from northern Honshu (NHo1 and NHo2). Six populations were monomorphic, i.e., harbored a single AFLP phenotype (Table 1), which could be due to both seed production through high inbreeding and clonal spread.

The neighbor-joining analysis (NJ, Fig. 2) illustrates a strong divergence of populations morphologically assigned to *C. longifructus*. *Cardamine scutata* itself was resolved in two main clusters (although lacking bootstrap support) in the NJ tree, as well as in the ordination space of PCoA, separated along the first axis (Fig. 3). One group encompassed populations mostly from the southern part of the range (Korea, Kyushu, Shikoku, southern and central Honshu; denoted as "S"), and the other comprised populations mainly from the northern part (Kamchatka, Hokkaido, northern and central Honshu; denoted as "N"; see Fig. 1). Most of the studied populations consisted of genetically similar individuals clustering together. Five populations, however, appeared to be highly heterogeneous (Hok6, Hok2, NHo3, NHo4, CHo8; highlighted in Figs. 1, 3), harboring genetically divergent individuals placed within both the N and S groups. A single population from southeastern Hokkaido (Hok8) contained individuals with intermediate phenotypes, placed in the center of the PCoA graph and at the base of the N group in the NJ tree (Figs. 2, 3).

Bayesian clustering was consistent with the above phenetic analyses. The BAPS optimal partition estimate showed five clusters (I–V) (Fig. 2). The N group of *C. scutata* was supported (cluster III) as distinct, but the Kamchatkan populations were resolved as a separate cluster (II) as well. The S group was split into two clusters (I, IV). Finally, three individuals with somewhat divergent AFLP profiles, placed at the base of the *C. scutata* cluster in the NJ tree, were resolved as a separate cluster (V). In the STRUCTURE analysis of *C. scutata*, mean $L(K)$ increased up to $K = 4$, but started to flatten out at $K = 3$. Replicate runs produced stable results with a coefficient of similarity of 1.0 only for $K = 2$, which showed a division between the N and S groups. Heterogeneity or intermediacy of certain populations (see above) was reflected in the proportional assignment of their individuals into the N or S clusters (see Fig. 2 and Appendix S1, see Supplemental Data with the online version of this article). Similarity among replicate runs was also reasonably high for $K = 3$; these replicates differed only in the assignment of two populations (Shi1, Kor). $K = 3$ resulted in the division of S into two clusters that corresponded closely to the BAPS partition (I and IV; Fig. 2 and online Appendix S1 at http://www.amjbot.org/cgi/content/full/ajb.0900361/DC1). At $K = 4$ and higher, only poor similarity among runs was achieved.

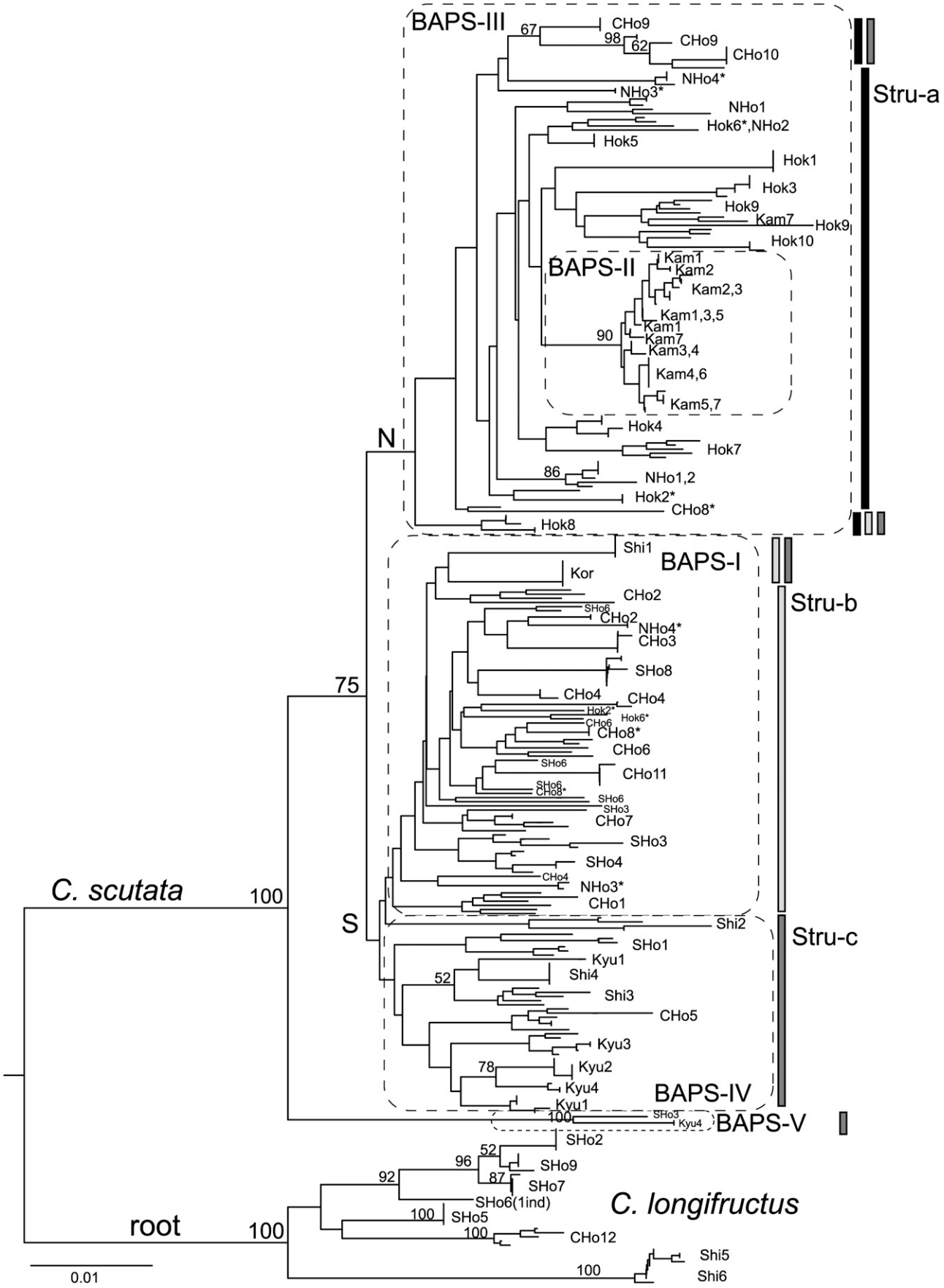
AMOVA (excluding heterogeneous populations as revealed by the STRUCTURE analyses) supported Bayesian clustering and revealed significant differentiation between the N and S groups, showing that 34.7% ($F_{CT} = 0.35$, df = 1, $P < 0.001$) of total variance was explained by the two groups, whereas 45.8% ($F_{SC} = 0.70$, df = 39, $P < 0.001$) of the variation was between populations within the two groups. When considering three groups (one N and two S groups, following the STRUCTURE output) the level of differentiation was similar (35.7% of total variance explained by the three groups, $F_{CT} = 0.36$, df = 2, $P < 0.001$; 43.9% of variance between populations within the groups, $F_{SC} = 0.68$, df = 35, $P < 0.001$).

The average number of AFLP fragments per individual was rather stable in *C. scutata* (N_{frag}/ind ± SD = 69.1 ± 4.4). Specific markers were observed for the N (41 markers) and S (70 markers) groups, but none of these markers was fixed (data from the heterogeneous populations mentioned above were excluded). Genetic diversity within populations varied widely, with D_{Nei} ranging 0–0.0826 (mean = 0.0225, SD = 0.0207) and P (%) ranging 0–17.71 (mean = 4.80, SD = 4.28). There was, however, no clear geographical pattern in the distribution of either genetic diversity or divergence (Table 1, online Appendix S1), and we speculate that this could be partly due to the low sample sizes. Geographical trends were seen only in the Kamchatkan populations, which had low diversity and DW1 values (except Kam7).

cpDNA diversity and differentiation—The trimmed 5′ part of the *trnL-trnF* alignment was 324 bp long. A region contain-

→

Fig. 2. Neighbor-joining tree based on AFLP data of *C. scutata*, rooted with the accessions of *C. longifructus*. Terminal labels refer to populations (see Table 1). Numbers above branches indicate bootstrap support greater than 50% (bootstrap values for terminal, population-specific branches are omitted). Genetically heterogeneous populations are marked with asterisks. Bayesian clustering is mapped onto the tree: frames labeled as BAPS-I through BAPS-V indicate the clusters inferred by BAPS; black, dark gray, and light gray vertical bars (Stru-a through Stru-c) show the clusters generated by STRUCTURE at $K = 3$ (two or three parallel bars indicate populations with uncertain assignments across the replicate runs).


BAPS-III
BAPS-II
BAPS-I
BAPS-IV
BAPS-V
Stru-a
Stru-b
Stru-c
N
S
C. scutata
C. longifructus
root
0.01
CHo9
CHo10
NHo4*
NHo3*
NHo1
Hok6*,NHo2
Hok5
Hok1
Hok3
Hok9
Kam7
Hok9
Hok10
Kam1
Kam2
Kam2,3
Kam1,3,5
Kam1
Kam7
Kam3,4
Kam4,6
Kam5,7
Hok4
Hok7
NHo1,2
Hok2*
CHo8*
Hok8
Shi1
Kor
CHo2
SHo6
CHo2
NHo4*
CHo3
SHo8
CHo4
CHo4
Hok2*
CHo6
Hok6*
CHo8*
CHo6
SHo6
CHo11
SHo6
CHo8*
SHo6
SHo3
CHo7
SHo3
SHo4
CHo4
NHo3*
CHo1
Shi2
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Kyu1
Shi4
Shi3
CHo5
Kyu3
Kyu2
Kyu4
Kyu1
SHo3
Kyu4
SHo2
SHo9
SHo7
SHo6(1ind)
SHo5
CHo12
Shi5
Shi6
67
98
62
86
90
75
100
52
78
100
52
96
87
92
100
100
100

ing three types of overlapping indels 2–13 bp long at about base pair position 100 was identified and coded as a single four-state character. Eleven unique sequence types (*trnL-trnF* haplotypes) were identified, which were distinguished by six (1.9%) polymorphic nucleotide sites. One additional haplotype was observed in three individuals from SHo3 and Kyu4 (Table 1), differing by another three polymorphic sites. The *rpl32-trnL*$^{(UAG)}$ alignment was 844 bp long, with one indel (5 bp) coded as a binary character. In total, 28 different haplotypes were detected, which were distinguished by 36 (4.3%) polymorphic nucleotide sites. Two additional haplotypes were observed in the same three individuals from SHo3 and Kyu4 (Table 1), differing by another 16 polymorphic sites. Indels due to poly N or microsatellite stretches (varying inconsistently with substitutions) were ignored. The combined alignment [*rpl32-trnL*$^{(UAG)}$ + *trnL-trnF*], which was used for further analyses, was thus 1168 bp long plus two coded indels and comprised 36 distinct haplotypes. All sequences have been deposited in GenBank with accession numbers GQ214988–GQ215059 and GQ405866–GQ405965 for *trnL-trnF* and GQ214816–GQ214987 for *rpl32-trnL*$^{(UAG)}$.

The haplotype network based on the combined cpDNA alignment revealed two haplotype lineages separated by six mutational steps, displaying clear geographic structure (Fig. 4). The lineages corresponded to the N and S groups resolved by the AFLP data. In each lineage, one frequent and widespread haplotype (denoted as S1 and N1, indicated by large symbols; Fig. 4A) was identified, resolved in an interior position. Within the S lineage, it was haplotype S1, detected in 13 populations from the southern regions (Korea, Shikoku, Kyushu, and southern and central Honshu), but found also in two populations in the north. As many as 19 haplotypes (S2–S20) were connected to S1 in a starlike network with one or two (rarely three) inferred mutations. All were restricted to southern regions, except of two haplotypes (S13, S17) detected in southwestern Hokkaido in two and one individual, respectively. Within the N lineage, haplotype N1 was dominant, found in 10 populations from the north (Kamchatka, Hokkaido, and northern Honshu). Another 13 haplotypes were observed within the N lineage, resolved mostly in derived positions, connected by one to four mutational steps either to N1 (N2–N8), or to another interior but unidentified (not sampled or extinct) haplotype (N9–N14). With exception of haplotypes N3 and N10, reaching central Honshu, all these haplotypes were found only in the north.

All haplotypes except for S1 and N1 were present at low frequencies; eight haplotypes (indicated by middle-large symbols; Fig. 4A) were found in two to three populations, often located in the same or adjacent regions, while the remaining haplotypes were restricted to single populations. Most populations (36 of 53, 68%) were fixed for single haplotypes, although if more individuals per population were analyzed, this number might be lower. Polymorphic populations, harboring two to three different haplotypes, were distributed across the study area. Eight of these harbored closely related haplotypes, but five populations contained individuals with clearly distinct haplotypes, i.e., from both the N and S lineages (Hok6, Hok2, NHo4, CHo8, CHo9). They coincided very well with the populations that appeared highly heterogeneous also in the AFLP data (see Table 1, Figs. 1, 4), located in central Honshu and northern Honshu–southwestern Hokkaido (Oshima Peninsula) region.

The coefficients of differentiation calculated for the total data set were high, especially when taking into account the similarities between haplotypes (N_{ST}; Table 2). The permutation test showed that N_{ST} is significantly greater than G_{ST} (which is based on the haplotype frequencies; $P < 0.01$), confirming a significant phylogeographical signal in the data. When considering the N and S lineages separately; however, N_{ST} values were not significantly greater than G_{ST}. This is apparent in the haplotype network, where both lineages comprised several haplotypes but with little structure; the starlike network was especially prominent in the S lineage (Fig. 4A). Partitioning of total genetic variance into hierarchical levels (AMOVA) was strongly affected by whether distances between haplotypes were taken into account or not. Compared to the analysis using haplotype frequencies only, the analysis based on pairwise haplotype distances revealed much higher differentiation between the N and S groups (72% vs. 21% percentage of variation) and lower within-group (23% vs. 65%) and within-population components (5% vs. 14%). This is in accordance with the above tests of $N_{ST} > G_{ST}$ and confirms a strong structure with related haplotypes more commonly found within populations and within groups.

Rooting the haplotype network based on the *trnL-trnF* sequences of *C. scutata* (figure not shown) with sequences of the closely related taxa *C. parviflora* and *C. pensylvanica* (following the phylogeny presented by Lihová et al., 2006) confirmed the ancestral position of haplotype S1 (Fig. 4A).

As mentioned, two distant haplotypes (marked with asterisks in Table 1, Fig. 4A) were detected in two populations, Kyu4 and SHo3. Individuals bearing these haplotypes also had somewhat distinct AFLP profiles, located at the base of the *C. scutata* cluster (Fig. 2). Comparisons with previously published sequences (on the basis of *trnL-trnF* data) indicate that they are closest to *C. pensylvanica* (accessions US37 and US42; see Lihová et al., 2006).

DISCUSSION

Phylogeographical history of Cardamine scutata—*Congruence of AFLP and cpDNA data*—Two distinct genetic groups are revealed congruently by AFLP and cpDNA data within *C. scutata*, which apparently reflect intraspecific lineages that have remained isolated from each other through long time periods, supposedly since (at least) the last glaciation. The boundary between these north–south differentiated groups is located between the Tohoku region of northern Honshu and the Chubu and Kanto regions of central Honshu (Figs. 1, 4). Furthermore, AFLP data identify two subgroups within the southern group, displaying additional geographic structuring in the SW–NE direction (Fig. 1). This differentiation is not strong; it is equivocal for some populations (Kor, Shi1; but higher sample sizes might have brought more resolution), and the assignment of the population CHo5 contradicts the main geographic trend. The cpDNA data, although exhibiting high polymorphism, do not indicate much regional substructure within either the northern or the southern lineage. Starlike structure and most haplotypes being rare, confined to 1–3 populations, indicates recent diversification and precludes the identification of subgroups or the tracking of colonization routes (Fig. 4).

Most populations of *C. scutata* appeared genetically homogeneous because individuals from the same populations often clustered together, and multiple individuals even shared the same AFLP phenotypes. This clearly supports our observations that selfing is the main breeding mode in this species, accompanied by some extent of clonal spread. The distribution of genetic

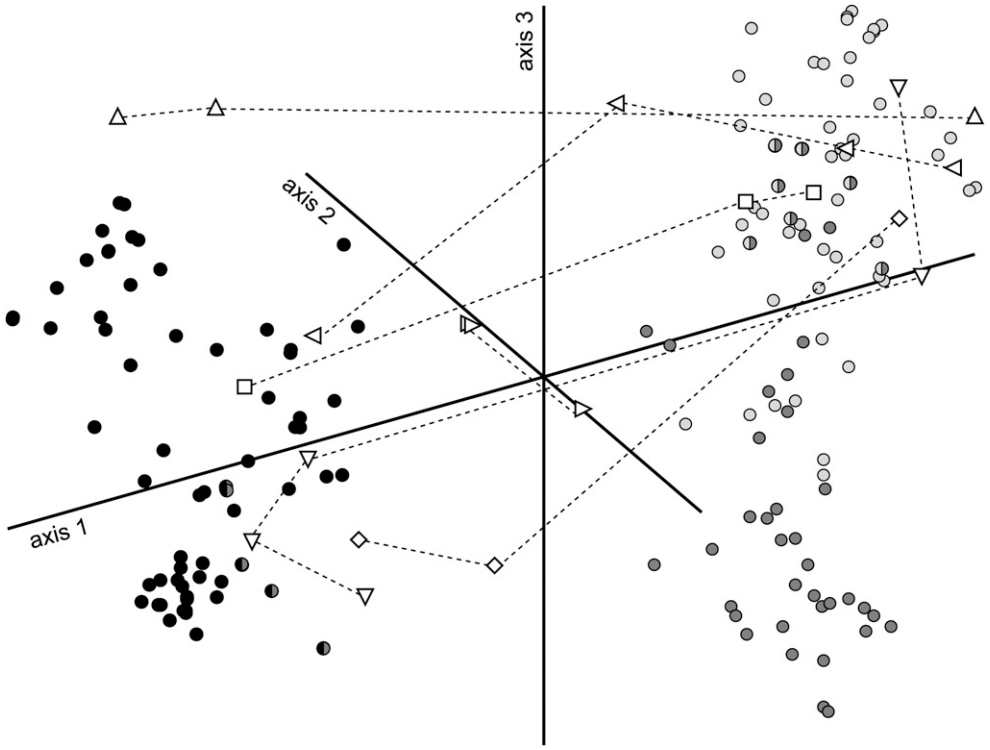

Fig. 3. Principal coordinate analysis of AFLP data from 268 *Cardamine scutata* individuals. The first three axes explain 32.0%, 9.2%, and 5.5% of the total variation. Population assignments into three clusters as inferred by Bayesian clustering (Fig. 2) are indicated by black (N group, Stru-a, BAPS-II+III), light gray (Stru-b, BAPS-I, S group) and dark gray (Stru-c, BAPS-IV, S group) circles; half-divided circles indicate individuals with uncertain assignments. Genetically heterogeneous populations are highlighted by different symbols (the dotted lines join individuals belonging to the same population): squares, NHo3; upward-pointing triangles, NHo4; left-pointing triangles, CHo8; diamonds, Hok2; downward-pointing triangles, Hok6. Right-pointing triangles indicate the genetically intermediate population Hok8.

diversity (D_{Nei}, P [%]) and rare markers (DW1 values) within populations, which may reflect long-term survival in refugia vs. recent colonization and establishment, does not show any geographically correlated pattern in the Japanese Archipelago (see online Appendix S1). If stepwise recolonization had occurred, we would expect a diversity gradient (Hewitt, 2000), which is not seen here. Still, due to high rates of gene flow (via both pollen and seeds) in many plant species, the empirical data often do not show such strong patterns. With the present data, we cannot infer the locations of refugia and possible routes of postglacial expansion in Japan. Admittedly, we can speculate that higher sample sizes may have brought somewhat more conclusive patterns. Nevertheless, reduced within-population diversity and low divergence is observed in all but one of the Kamchatkan populations, suggesting relatively recent colonization of this area, coupled with founder effects resulting from long-distance dispersal events. Kamchatkan populations form a distinct genetic cluster, embedded within the northern lineage. No specific cpDNA haplotypes are observed here, also supporting the scenario of recent establishment from northern Japan. Importantly, populations Kam2 and Kam3 are located near the northernmost records of this species, which diminishes the possibility of additional biogeographical links via a northern route (NE mainland Russia or Beringia). Only one population (Kam7) exhibits slightly higher variation; it is polymorphic for the cpDNA marker, and the AFLP profile of one individual corresponds to those from Hokkaido (Fig. 2). This pattern is attributable to a recent long-distance dispersal event. It has been reported that some birds that migrate between northern Honshu and Russian Far East, such as *Cygnus columbianus* and *Anser fabalis middendorfi*, feed on *C. scutata* in paddy fields (Watanabe, 2008). These migrating birds may have contributed to the long-distance dispersal, at least for northern part of the distribution range of *C. scutata*, by carrying mud that contains seeds. Similarly, colonization of Kamchatka from Hokkaido has been inferred for *Juniperus communis*, most likely mediated by migrating birds (Adams et al., 2003). Several marine straits separate the Kuril Islands from each other and from Kamchatka, and many of these straits remained open during the decline in sea level of the last glaciation (Pietsch et al., 2003). Still, occasional long-distance dispersal events and stepwise colonization may have played a major role.

The cpDNA network is, according to coalescent theory, highly consistent with a scenario of recent diversification and demographic expansion (Hudson, 1990). A high number of rare haplotypes are identified, all resolved in derived positions and connected to ancestral haplotypes directly by few mutational steps. This structure, implying rapid radiation, is most conspicuous in the southern lineage (Fig. 4). A similar pattern of diversification, attributed to expansion during climate warming in an interglacial period, has been observed in *Arabidopsis thaliana* (Beck et al., 2008). Unlike that study, we are not able to infer the ancestral area of *C. scutata*. Inferred ancestral haplotypes (S1, N1) are widespread across their northern or southern ranges, indicating efficient and rapid spread across the area. The diversification that we currently observe in the Japanese

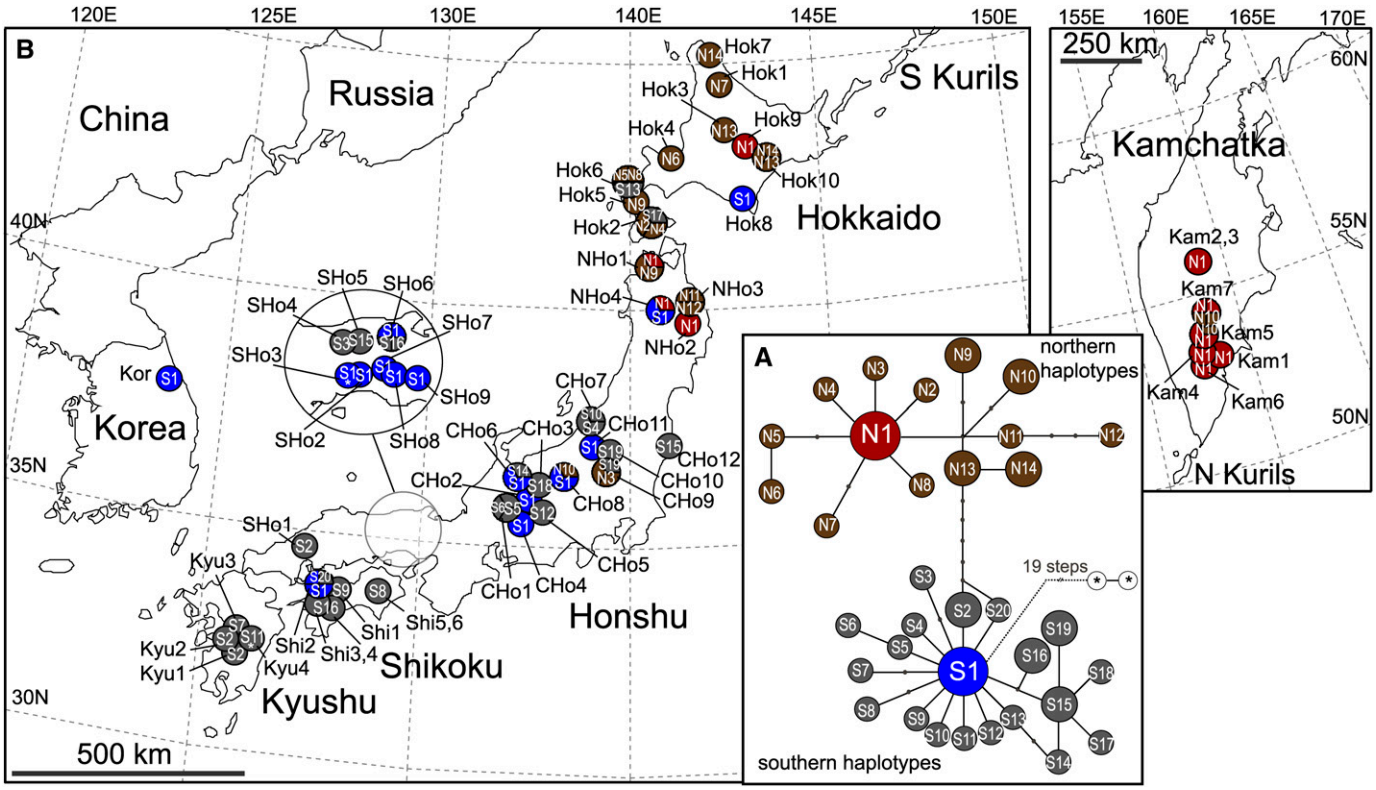

Fig. 4. Parsimony network of cpDNA haplotypes based on the combined *rpl32-trnL* + *trnL-trnF* sequence data from *Cardamine scutata* and their geographic distribution. (A) In the haplotype network, the two large circles indicate the dominant haplotypes—the northern haplotype N1 (in red) and the southern haplotype S1 (in blue); eight middle-large circles (N9, N10, N13, N14, S2, S15, S16, S19) indicate haplotypes present in two to three populations. The group of northern haplotypes (N2-N14) is shown in brown and the group of southern haplotypes (S2-S20) in gray. Two white circles with asterisks inside denote more distant haplotypes (see the text). Lines indicate single mutational steps; dots represent haplotypes not found in any accession. (B) In the map, each circle indicates one population represented by three to four individuals. Single-haplotype circles show monomorphic populations; circles of two to four divisions show the proportion of different haplotypes detected in a given population (see also Table 1).

Archipelago apparently occurred relatively recently and within the established populations, or shortly before their establishment, since a vast majority of derived haplotypes were restricted to single populations (or a few adjacent ones; Fig. 4). In future it will be interesting to explore variation of the *rpl32-trnL*$^{(UAG)}$ intergenic spacer also in other Brassicaceae relatives and to estimate its mutation rate. That would allow dating the divergence between the southern and northern intraspecific lineages of *C. scutata* and also placing the start of the demographic expansion in time. Although the occurrence of populations of *C. scutata* in mainland Asia appears very restricted nowadays (see introduction), it remains an open question if they were more widespread in the past and what role they played in the Pleistocene survival. Analyzing populations from Sakhalin and mainland Russia in future may complete the picture observed here.

Comparison with other phylogeographical patterns in Japan and the role of land bridges—The main pattern of north–south genetic differentiation that we have found in *C. scutata* has been observed in several other plant species from the Japanese Archipelago. The distribution of two haplotype groups resolved within the cold-adapted and submerged herbaceous species of *Ranunculus* subgen. *Batrachium* coincides very well with the two lineages of *C. scutata* and even displays a strikingly similar boundary region (Koga et al., 2008). Similar geographical division has been inferred in *Quercus mongolica* var. *crispula* (Okaura et al., 2007), *Betula maximowicziana* (Tsuda and Ide, 2005), and several alpine species (e.g., Fujii and Senni, 2006; Ikeda et al., 2008a). Palynological data indicate that oaks almost disappeared from Hokkaido and northern Honshu during the last glacial maximum, while they were common in central and southwestern Japan. In the postglacial period, conversely, they retreated to higher altitudes in southwestern Japan. Genetic data also supports the conclusion that the Quaternary population history of this cool temperate tree differed between the northeastern and southwestern parts of Japan (see Okaura et al., 2007). In alpine plants, forest expansion during warm interglacial periods caused range contraction and retreat into high-elevation refugia, while glacial periods allowed range expansions (Ikeda et al., 2008b).

Overall congruence in the north–south genetic differentiation within species of different life forms, dispersal modes, and habitat preferences, for which different (post-) glacial histories are expected, is rather surprising. We assume, however, that different processes and factors may have caused this similarity, as pointed out by Fujii et al. (1999) and Fujii and Senni (2006) for alpine species. First, a long-term barrier to gene flow and population expansion across the boundary region could be considered; second, two colonization routes from the Asian continent via a southern (Korean Peninsula) and northern land bridge (Sakhalin) can be assumed; or third, two colonization events (in different glacial periods) may have occurred via a single route.

For *C. scutata*, we consider the first two scenaria to be likely, and they may have acted in concert.

The mountain ranges of the Chubu district, running from the Pacific coast to the Sea of Japan and reaching 2000–3000 m a.s.l. (see Fig. 1), may indeed represent a geographic barrier for *C. scutata*. This species is rarely found in high altitudes of subalpine or alpine belts (H. Kudoh, personal observervation), and these mountains certainly reduce the extent of gene flow across them. The effect of this barrier was probably much stronger during glacial periods, when populations may have been pushed to more favorable, warmer, and more humid sites even at lower altitudes than today. But occassionally, the mountain ranges may have been permeable during interglacial and the postglacial period, allowing secondary contacts between previously isolated lineages. At the same time, the decline in sea level during the glacial periods created several land bridges or significantly reduced the width of marine straits (Japan Association for Quaternary Research, 1977; Ohshima, 1990), allowing dispersal between islands and to or from continental Eurasia. Therefore, we hypothesize that Japanese populations of *C. scutata* may have been in contact with those on the continent via both the northern and southern routes. In the south, Honshu, Shikoku, and Kyushu were connected to a single unit. Although the deep Tsushima and Korean straits separating them from Korea were not completely closed during the last glaciation, they may have not been too wide to prevent seed dispersal via birds, water, or wind (see, e.g., Yamaji et al., 2007). Indeed, these connections are reflected in our data; populations from southern and central Honshu, Shikoku, Kyushu, and Korea are genetically very similar. In the north, the Tsugaru strait separating Honshu from Hokkaido remained open during the last glaciation, but its width was reduced, and it was apparently not a serious dispersal barrier. Strong genetic affinity between populations from northern Honshu and Hokkaido is seen not only in the current study, but also in several others (Fujii and Senni, 2006, Okaura et al., 2007; Koga et al., 2008). Hokkaido, in turn, was continuously connected to the Asian mainland via Sakhalin during the last glacial period, allowing extensive plant dispersal from and to the continent (see, e.g., Aizawa et al., 2009). We lack samples of *C. scutata* from Sakhalin and the Russian coast, so this issue cannot be addressed in greater detail.

Secondary contacts and within-population heterogeneity—The two genetic lineages of *C. scutata* do not display a sharp division line but a rather broad boundary zone with some overlap at both the regional and population scales. Some of the southern haplotypes extend beyond the boundary region of central/northern Honshu and are found as far north as southern Hokkaido, while some northern haplotypes are found as far south as the inland regions of central Honshu. We may speculate that the northern lineage might be better adapted to the more continental climate in the center of Honshu and became established there successfully. Genetically heterogeneous populations are thus observed in central and northern Honshu and in southern Hokkaido, suggesting secondary contact between the diverged lineages at their distribution limits, i.e., in the boundary zone, but recent long-distance dispersal events may have also contributed to the present pattern (e.g., the case of N10 haplotype present in Kamchatka and central Honshu). Apparently, the seeds of *C. scutata*, although lacking special long-distance dispersal adaptations, can occasionally overcome large distances by means of birds, wind, or water flow. Interestingly, both AFLP and cpDNA markers were highly consistent in showing genetic admixture within the same populations. The AFLP phenotypes found in most of these populations have remained distinct, falling into the clearly different N and S clusters. This finding is attributable to the predominance of selfing with some clonal spread, as already noted. Within-population heterogeneity, with the absence of intermediate AFLP phenotypes, has also been reported in other predominantly inbreeding species: *Cardamine resedifolia* (Lihová et al., 2009), *Comastoma tenellum* (Schönswetter et al., 2004), and *Veronica alpina* (Albach et al., 2006). In such species, gene flow via pollen is limited. Therefore, after seeds from a distant population arrive, genetically distinct individuals are produced, and this distinctiveness may persist over generations. Still, all five individuals examined in population Hok8 and a few individuals from the other two populations (Hok2, CHo8) display intermediate phenotypes (Fig. 3), suggesting a certain level of outcrossing.

Conclusions—Our study shows the presence of two intraspecific lineages within the studied perennial herb *Cardamine scutata*. Interestingly, this pattern of north–south genetic differentiation is rather common among several other plant species studied in Japan (e.g., Koga et al., 2008; Tsuda and Ide, 2005), despite their distinct biological features. We assume that different processes and factors may have brought about this similarity, and future studies may reveal more details of individual species' histories. Both AFLP and cpDNA markers employed here clearly supported the same main pattern, and although AFLPs did not show additional diversity patterns indicative of range changes or specific colonization routes, cpDNA variation implied recent (presumably postglacial) diversification and demographic expansion within both intraspecific lineages. It is also apparent that species' life history traits such as the efficient seed dispersal, prevalence of selfing, and the ability of clonal spread, have significantly shaped its genetic structure in conjunction with extrinsic factors and historical events. We also believe that disentangling the polyploid origin of *C. scutata* in future studies may shed further light into the evolutionary history of this species.

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APPENDIX 1. Collection information for 46 populations of *Cardamine scutata* sampled and analyzed in this study. Herbarium vouchers are deposited in the herbarium of the Institute of Botany, SAS, Bratislava, Slovakia (SAV) and in the herbarium of Shoei Junior College, Kobe, Japan. Collector abbreviations: *JL*, J. Lihová; *KM*, K. Marhold; *HK*, H. Kudoh.

Population code; Locality, Altitude, Collection date, *Collector*; *Collection code of the vouchers*.

Kor; S Korea, Gangwon-do, Mt. Odaesan, Wolcheong Temple, 720 m, 15-Jun-05, *HK, KM, JL* et al.; *K5*. **Kyu1**; Japan, Kyushu, Kumamoto prefecture (pref.), Sagara-mura, Shiura, 425 m, 7-May-06, *HK, K. Hirose, T. Shiga*; *H0038*. **Kyu2**; Japan, Kyushu, Kumamoto pref., Itsuki-mura, Otsu, 600 m, 8-May-06, *HK, K. Hirose, T. Shiga*; *H0047*. **Kyu3**; Japan, Kyushu, Kumamoto pref., Misato-machi, Hayakusu, 820 m, 8-May-06, *HK, K. Hirose, T. Shiga*; *H0063*. **Kyu4**; Japan, Kyushu, Miyazaki pref., Gokase-cho, Mt. Shiraiwayama, 970 m, 14-Jun-04, *HK, KM*; *JP86/04*. **Shi1**; Japan, Shikoku, Ehime pref., Omogo-mura, Kuromori-toge, 900 m, 16-Jun-04, *HK, KM*; *JP93/04*. **Shi2**; Japan, Shikoku, Ehime pref., Matsuyama-shi, Tsuenofuchi-kohen (in cultivation), 50 m, 12-Jun-04, *HK, KM*; *JP84/04*. **Shi3**; Japan, Shikoku, Ehime pref., Seiyo-shi, Nomura-cho, Komatsu, 250 m, 9-May-06, *HK, K. Hirose, T. Shiga*; *H0068*. **Shi4**; Japan, Shikoku, Ehime pref., Seiyo-shi, Nomura-cho, Yokobayashi, 245 m, 9-May-06, *HK, K. Hirose, T. Shiga*; *H0065*. **SHo1**; Japan, Honshu, Hiroshima pref., Akiohta-cho, Nakatsutsuga, Ini, 440 m, 17-Feb-07, *HK*; *H07-001*. **SHo3**; Japan, Honshu, Hyogo pref., Tatsuno-shi, Shinguu-cho, Okugoya, 255 m, 22-May-06, *HK, K. Hirose*; *H0084*. **SHo4**; Japan, Honshu, Hyogo pref., Shisou-shi, Haga-cho, Akazai valley, 645 m, 15-May-06, *HK, K. Hirose*; *H0074*. **SHo6**; Japan, Honshu, Hyogo pref., Asago-shi, Wadayama-cho, Itoi valley, 425 m, 17-Apr-06, *HK, K. Hirose*; *H0017*. **SHo8**; Japan, Honshu, Hyogo pref., Taka-cho, Naka-ku, Makino, 195 m, 26-Apr-06, *HK, K. Hirose*; *H0027*. **CHo1**; Japan, Honshu, Gifu pref., Gujyo-shi, Hachiman-cho, Ohora-dani, 400 m, 21-Aug-07, *HK*, *T. Kawagoe*, *M. Yamaguchi*; *H07-825*. **CHo2**; Japan, Honshu, Nagano pref., Kiso-cho, Kaida-kogen, Takatsubo, 1280 m, 22-Aug-07, *HK*, *T. Kawagoe*, *M. Yamaguchi*; *H07-829*. **CHo3**; Japan, Honshu, Nagano pref., Azumi-mura, Kamikochi, 1540 m, 13-Jul-03, *HK, KM, JL*; *JP88*. **CHo4**; Japan, Honshu, Gifu pref., Ena-shi, Osashima-cho, Kusimi, 260 m, 26-Sept-06, *HK*; *H06-332*. **CHo5**; Japan, Honshu, Nagano pref., Komagane-shi, Komagane-kogen, 960 m, 26-Sept-06, *HK*; *H06-333*. **CHo6**; Japan, Honshu, Toyama pref., Toyama-shi, Inotani, Tokonijinotaki Fall, 250 m, 28-Sept-06, *HK*; *H06-344*. **CHo7**; Japan, Honshu, Gunma pref., Takayama-mura, Nakayama, 610 m, 27-Sept-06, *HK*; *H06-340*. **CHo8**; Japan, Honshu, Nagano pref., Sanada-cho, Osa, Ohhinata, 880 m, 27-Sept-06, *HK*; *H06-339*. **CHo9**; Japan, Honshu, Tochigi pref., Nikko-shi, Chynzenji lake, Senjyugahama, 1290 m, 6-Jul-03, *HK, KM, JL*; *JP81*. **CHo10**; Japan, Honshu, Tochigi pref., Nikko-

shi, Sotoyamazawa River, 1410 m, 24-Jun-04, *HK, KM*; *JP100*. **CHo11**; Japan, Honshu, Niigata pref., Yunotani-mura, Ginzandaira, 915 m, 22 & 23-Jun-04, *HK, KM*; *JP98*. **NHo1**; Japan, Honshu, Aomori pref., Kanagi-cho, Takahashi-zawa River, 140 m, 30-Jun-04, *HK, KM*; *JP107*. **NHo2**; Japan, Honshu, Iwate pref., Iwaizumi-cho, Okawa River, Hitsutori-shitsugen, 985 m, 27-Jun-04, *HK, KM*; *JP103*. **NHo3**; Japan, Honshu, Iwate pref., Kuzumaki-cho, Mt. Sode-yama, Mabechi-gawa River, 915 m, 27-Jun-04, *HK, KM*; *JP102*. **NHo4**; Japan, Honshu, Akita pref., Kazuno-shi, Hachimantai, Onuma pond, 950 m, 21-Jun-05, *HK, KM, JL*; *JP132*. **Hok1**; Japan, Hokkaido, Nakagawa-gun, Bifuka-cho, Panke, 400 m, 6-Jun-05, *HK, KM, JL*; *JP66/05*. **Hok2**; Japan, Hokkaido, Kamiiso-gun, Kamiiso-cho, Moheji, 100 m, 2-Jun-05, *HK, KM, JL*; *JP109*. **Hok3**; Japan, Hokkaido, Kamikawa-gun, Higashukagura-cho, Chiyogaoka, 250 m, 5-Jul-04, *HK, KM*; *JP119*. **Hok4**; Japan, Hokkaido, Otaru-shi, Asari-touge Pass, 670 m, 6-Jul-04, *HK, KM*; *JP122*. **Hok5**; Japan, Hokkaido,Yamakoshi-gun, Yakumo-cho, Namarikawa, 220 m, 2-Jun-05, *HK, KM, JL*; *JP49/05*. **Hok6**; Japan, Hokkaido, Setana-gun, Setana-cho, Kariba-keikoku, 200 m, 3-Jun-05, *HK, KM, JL*; *JP50/05*. **Hok7**; Japan, Hokkaido, Souya-gun, Sarufutsu-mura, Sarufutsu river, 20 m, *JP69/05*. **Hok8**; Japan, Hokkaido, Urakawa-gun, Urakawa-cho, Syuman river, 70 m, 4-June-05, *HK, KM, JL*; *JP128*. **Hok9**; Japan, Hokkaido, Katou-gun, Kamishihoro-cho, Shiishikaribetsu-gawa River, 690 m, 2-Jul-04, *HK, KM*; *JP113*. **Hok10**; Japan, Hokkaido, Nakagawa-gun, Honbetsu-cho, Honbetsu-gawa River, 130 m, 11-Aug-07, *HK, K. Watanabe*; *JP07-814*. **Kam1**; Russia, Kamchatka, Elizovskii raion, NE of Elizovo, 120 m, 4-Jul-07, *KM, V. Yakubov*; *EL3*. **Kam2**; Russia, Kamchatka, Bystrinskii raion, Esso, Ulavkavchan river, 470 m, 8-Jul-07, *KM, HK, K. Shimizu, V. Yakubov*; *UKC*. **Kam3**; Russia, Kamchatka, Bystrinskii raion, Esso, Bystra river, 420 m, 7-Jul-07, *KM, HK, K. Shimizu, V. Yakubov*; *ESSO6*. **Kam4**; Russia, Kamchatka, Elizovskii raion, Nachikinskoe ozero lake, 405 m, 5-Jul-07, *KM, V. Yakubov*; *NAC2*. **Kam5**; Russia, Kamchatka, Mil'kovskii raion, Pushchino, Pravaya Kamchatka river, 500 m, 6-Jul-07, *KM, HK, K. Shimizu, V. Yakubov*; *KAM*. **Kam6**; Russia, Kamchatka, Elizovskii raion, Nachiki, 350 m, 10-Jul-07, *KM, V. Yakubov*; *NAT*. **Kam7**; Russia, Kamchatka, Mil'kovskii raion, Pushchino, Denokhonok river, 265 m, 9-Jul-07, *KM, HK, K. Shimizu, V. Yakubov*; *DEN*.