Title:

Habitat Segregation and Genetic Relationship of Two Heptageniid Mayflies *Epeorus latifolium* and *Epeorus l-nigrus* in the Shinano-gawa River Basin

Authors:

Masaki Ogitani[1)], Kazuki Sekiné[2)] and Koji Tojo[3, 4)]

Affiliations:

1) Department of Environmental System Science, Graduate School of Science and Technology, Shinshu University

2) Department of Mountaine and Environmetal Science and Technology, Shinshu University

3) Department of Biology, Faculty of Science, Shinshu University

4) Institute of Mountain Science, Shinshu University

Addresses

1-4) Asahi 3-1-1, Matsumoto, Nagano 390-8621, Japan

Abbreviated title:

Habitat Segregation and Genetic Relationship of Two Heptageniid Mayflies

Corresponding author:

Koji TOJO

Department of Biology, Faculty of Science, Shinshu University, Asahi 3-1-1, Matsumoto, Nagano 390-8621, Japan

TEL: +81-263-373341

FAX: +81-263-372560

E-mail address: ktojo@shinshu-u.ac.jp

Abstract

Heptageniid mayflies *Epeorus latifolium* and *Epeorus l-nigrus* are often the dominant species in the upper and midstream areas of Japanese rivers, as such they play a significant role in river ecosystems. However, although these two species have able to been identified using the morphological characteristics of the male in its adult stage, it is impossible to differentiate them in their nymphal stage. We have conducted a study to elucidate their distribution pattern, *i.e.*, the current distribution of these two species as in the Shinano-gawa River basin, based on quantitative field sampling and genetic analysis of nymphs and also some male adults of which it was able to reliably differentiate between the two species. From the data collected from the 30 study sites of one year long study, it is revealed that the *E. latifolium* and/or *E. l-nigrus* mayflies are clearly distributed over a very broad area, and they appeared to be the dominant species at about a third of the study sites. Based on our genetic analysis, including several male adult specimens of *E. latifolium* and *E. l-nigrus*, it was clearly revealed that *E. latifolium* and *E. l-nigrus* are respectively form two separate monophyletic clades. That is, *E. latifolium* and *E. l-nigrus* are clearly genetically differentiated, and it is considered that they each represent a discrete species. Then, we plotted the collection sites of reliably identified specimens of *E. latifolium* and *E. l-nigrus* on the Shinano-gawa River basin map. This resultant map clearly displays that *E. latifolium* is distributed in the upper stream area rather than *E. l-nigrus*. To conclude, a pronounced 'habitat segregation' or 'current distribution' is clearly observable.

Key Words: Heptageniidae, population structure, genetic structure, current distribution, mitochondrial DNA, COI

Introduction

Heptageniid mayflies *Epeorus latifolium* Uéno and *Epeorus l-nigrus* Matsumura are widely distributed throughout the rivers of Japan [*E. latifolium* inhabiting the Hokkaido, Honshu, Shikoku and Kyushu Islands, and also inhabit the Korean Penisula, Manchu of China and Far East Russia; *E. l-nigrus* inhabiting the Hokkaido, Honshu, Shikoku and Kyushu Islands (Yoon and Bae 1984; Bae et al. 1994, 2000; Bae and Yoon 1997; Bae 1997; Kluge 2004; Ishiwata and Takemon 2005)]. They are also often the dominant species in upper and midstream areas, as such they play a significant role in river ecosystems. However, although these two species can be identified using the morphological characteristics of the male in its adult stage, it is impossible to differentiate them in their nymphal stage (Ishiwata and Takemon 2005). To date, a lot of research and many studies have been conducted based upon benthic faunal river surveys (e.g., the national project "National Censuses on River Environments" conducted by the Japanese Ministry of Land Infrastructure and Transport, and some self-governing bodies). In these research and studies, some specimens have been treated as a "*E. latifolium*", when in fact in many cases the samples may have contained many "*E. l-nigrus*", because the nymphal illustration and descriptions of the species *E. l-nigrus* have not been listed in any publication to date (Ishiwata and Takemon 2005; Inada 2007).

A previous study of note, although based on little data (using 3 male specimens of *E. latifolium* and 11 male specimens of *E. l-nigrus*), reported the current distribution of each of these two mayflies, based upon male adult specimens from the Ibo-kawa River, Hyogo Prefecture (Inada 2007). According to this report, *E. latifolium* predominantly inhabited more upstream areas, while *E. l-nigrus* predominantly inhabited midstream areas. Other than this report, studies into the distribution of these two species are virtually none.

From this background and in order to understand these species more thoroughly, we have conducted a study to elucidate their distribution pattern, *i.e.*, the current distribution of these two species as in the Shinano-gawa River basin (main river and its comparatively large tributaries). The study was based on quantitative field sampling and genetic analysis of nymphs and also some male adults by which we can reliably differentiate between these two species.

Materials and Methods

Study sites

This study was conducted at 30 sites in the Shinano-gawa River basin (Table 1, Fig. 1), including eleven sites along the main Chikuma-gawa River (C1-11), 4 sites along the Sai-gawa River which is a direct tributary of the Shinano-gawa River (S1-4), and 4 sites along the Azusa-gawa River (A1-4), 6 sites in the Narai-gawa River (N1-6) and 5 sites in the Takase-gawa River (T1-5), and these 3 are each tributaries of the Sai-gawa River.

Sampling and observation

Mayfly nymphs were collected from each study site mentioned above by random sampling four times each season, throughout the year, from July 2008 to August 2009. Samples at each of the field research site were randomly taken during a specific time frame (20 minutes) with a D-flame hand net (mesh size: 1mm). All specimens collected were fixed with pure or highly concentrated (>80%) ethanol, and all heptageniid mayflies were chosen, sorted, identified and counted. In the nymphal stage, it is difficult to differentiate between the two species *Epeorus latifolium* and *Epeorus l-nigrus*. Therfore, we treated them as are group '*E. latifolium* and/or *E. l-nigrus*' without making a distinction between them.

Based on this sampling data, the numbers of *E. latifolium* and/or *E. l-nigrus* mayflies of all the collected heptageniid mayflies were calculated, at each study site. We examined the *E. latifolium* and/or *E. l-nigrus* mayflies' distribution patterns and trends.

Genetic analyses

In addition to our survey, we performed a genetic analysis of the '*E. latifolium* and/or *E. l-nigrus*' mayfly nymphs which were collected for quantitative sampling at each study site (as per the methods mentioned above). We also conducted a genetic analysis of some of the male adults, which can be reliably differentiated between the two species [3 males of E. *latifolium* collected at the Narai-gawa River (GenBank accession number: AB538379-5387381), and 6 males of *E. l-nigrus* collected at the Narai-gawa (AB538373-538375) and Azusa-gawa River

(AB538376-538378)]. Two *Epeorus aseculus* nymphs were also subjected to molecular phylogenetical analyses as an outgroup (AB538383-538384).

Total DNA was extracted from the specimens fixed with pure or highly concentrated ethanol, and purified using a DNeasy^{R} Tissue Kit (QIAGEN, Hilden). The COI genes were amplified by a PCR method using the primer sets (Folmer et al. 1994): LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). PCR products were purified with a Microcon^{R} Kit (MILLIPORE, Massachusetts). The purified DNA was sequenced directly by an automated method using an DYEnamic^{TM} ET Terminator Cycle Sequencing Kit (GE Healthcare UK, Buckinghamshire) on an automated sequencer (ABI PRISM 377 Genetic Analyzer; Perkin Elmer/Applied Biosystems, California). The COI sequence data of 98 nymphal specimens from 30 populations, 6 male specimens of *E. latifolium* and 3 male specimens of *E. l-nigrus*, have been submitted to the DNA data Bank of Japan (DDBJ database). The GenBank accession numbers are listed in Table 1 (the accession numbers of male adults and the outgroup are mentioned above).

All sequence data were aligned automatically with Clustal W (Thonpson et al. 1994) and MEGA version 4 (Tamura et al. 2007; Kumar et al. 2008), and then cross-checked by eye carefully and using CLC Workbench software (CLC bio, Aarhus). Haplotype diversity (h) and nucleotide diversity (π) and its variance and standerd deviation ere calculated for each river and river group using the software DnaSP v. 4.10 (Rozas et al. 2003). The distribution of genetic variance at different geographical levels was estimated by an analysis of molecular variance (AMOVA) using Arlequin 3.01 (Excoffier et al. 2005).

Phylogenetic analyses were performed by the neighbor-joining (NJ) method (Saitou and Nei 1987), implemented using the software PHYLIP version 3.57 (Felsenstein 1995), and the maximum parsimony (MP) method, implemented using the software MEGA version 4. Gaps and ambiguous sites were omitted from data set of phylogenetic analyses. The NJ analyses employed matrices of genetic distances generated using Kimura's two-parameter method (Kimura 1980), and confidences of branches were assessed by 1,000 bootstrap resamplings. The MP analyses were performed for all tree searches using in the program MEGA for a heuristic search, and the majority rule consensus tree method. Bayesian analysis as conducted four

times (independent runs started from different, randomly chosen trees) for each set of phylogenetic data using MrBayes (Huelsenbeck and Ronquist 2001). The Baysian analysis was inferred for COI [GTR+G substitution model was selected a the best substitution model based on the hLRT; 5,000,000 generations], and nodal support was assessed by posterior probabilities estimated from the final 80% sampled trees.

Results and Discussion

Current distribution of E. latifolium and E. l-nigrus

From the data collected from the 30 study sites in our year long study, it was revealed that the *E. latifolium* and/or *E. l-nigrus* mayflies were clearly distributed over a very broad area in the Shinano-gawa River basin [At only one study site (N2 site in the Narai-gawa River), were no species of *E. latifolium* or *E. l-nigrus* collected throughout the four seasons], and they appeared to be the dominant species at about a third of the study sites (Fig. 2). Especially, in 7 study sites, *E. latifolium* and/or *E. l-nigrus* was the strongly dominant species, accounting for more than 50% of the all heptageniid mayflies (C7 and C8 sites of the Chikuma-gawa River, S1, S3-4 sites in the Sai-gawa River, N6 in the Narai-gawa River, and the A4 site in the Azusa-gawa River).

Based on our genetic analysis (including several male adult specimens which we were able to reliably differentiate) of *E. latifolium* and *E. l-nigrus*, it was clearly revealed that they form two separate monophyletic clades (Fig. 3). “Clade I” corresponds to the species *E. latifolium*, and the “clade II” must be corresponding to the species *E. l-nigrus*. Furthermore, the monophyly of these two clades of *E. latifolium* and *E. l-nigrus* was strongly supported. The bootstrap proportions (BPs) and the Bayesian posterior probabilities [BPP] of *E. latifolium* were 99% (NJ), 99% (MP), 100% (Bayes), and the Bps/BPP of *E. l-nigrus* were 100% (NJ), 99% (MP), 99% (Bayes). That is, *E. latifolium* and *E. l-nigrus* were clearly genetically differentiated, and it is considered that they each represent a discrete species, even though all of their morphological characteristics during their nymphal stages are indistinguishable. The average genetic distance (*p*-distance) between *E. latifolium*

and *E. l-nigrus* for COI gene was approximately 3.5%. Applying the generalized arthropodan molecular clock of 1.4-2.6% sequence divergence per million years (e.g., Knowlton and Weigt 1998; Queck et al. 2004; Hou et al. 2007), we estimate that these species diverged 1.4-2.6 million years ago.

From the results of our genetic analyses, we plotted the collection sites of the reliably identified specimens of *E. latifolium* and *E. l-nigrus* on a Shinano-gawa River basin map (Fig. 4). This map clearly shows that *E. latifolium* is distributed in the upper stream area more than *E. l-nigrus*. At only two study sites (A4 site of the Azusa-gawa River, and N4 site of the Narai-gawa River), were both *E. latifolium* and *E. l-nigrus* collected together. Therefore, pronounced 'habitat segregation' or 'current distribution' was clearly observable. In the Chikuma-gawa main River, *E. latifolium* inhabited sites above ca. 1,400m altitude. As for the other rivers, in the Narai-gawa River, *E. latifolium* inhabited sites above ca. 795m alt., in the Takase-gawa River above ca. 800m alt., and in the Azusa-gawa River above ca. 610m alt.

Our results are strengthen those of a previous report by Inada (2007), which showed a similar tendency of these two heptageniid mayflies toward habitat segregation in the Ibo-kawa River (Hyogo Prefecture) based on the distribution data of a small number of males (i.e., 3 males of *E. latifolium* and 11 males of *E. l-nigrus* from four sites) of a single river, the Ibo-kawa River. However, in this study, a large number of specimens (i.e., more than 100 individuals) were used from five different rivers in the Shinano-gawa river basin, and furthermore genetic analysis (DNA bar-coding analysis) was conducted. As a result, it is considered that there is a very strong tendency toward habitat segregation between *E. latifolium* and *E. l-nigrus* as observed in the Shinano-gawa River basin and the Ibo-kawa River. This is highly likely considered to be the general pattern. The altitudes of the two species' boundaries differed between the four rivers. In particular, the elevations of the boundary distribution of the two species in the Narai-gawa, Takase-gawa, and Azusa-gawa Rivers (ca. 600-900m) were much lower than the main stream of the Chikuma-gawa River (>1,400m). We assume this interesting result is due to the amount of snow (precipitation) in winter. From the "AMDAS data (Japan Meteorological Agency)" for the precipitation of the AMDAS observatory sites near the study sites of our research, the average values based on the World Meterological

Organization (*i.e.*, 30- year average from 1971 to 2000) in winter (from December to February) and winter-spring (from March to May) at an upper stream area of the Chikuma-gawa River are respectively 132.1mm and 327.7mm (the Nobeyama AMDAS station), and 220.5mm and 483.8mm at an upper stream area of the Narai-gawa River (the Kisohirasawa AMDAS station), 482.4mm and 636.8mm at an upper stream area of the Azusa-gawa River (the Kamikochi AMDAS station), and 206.5mm and 310mm at an upper stream area of the Takase-gawa River (the Omachi AMDAS station). That is, the Narai, Azusa and Takase-gawa Rivers flow from the 'Japan Alps', one of the highest mountain ranges and a heavy snow area. It is thought that the effect of cold water due to snowmelt continues until late spring or early summer (although there is no firm data on water temperature in these rivers), and then, the cold water-adapted *E. latifolium* is able to inhabit lower elevation areas.

Effectiveness of genetic analysis for understanding the population structures of aquatic insects

In this study, we showed clearly the current distribution pattern of the related heptageniid mayflies *Epeorus latifolium* and *E. l-ngrus*. From figure 3, it was revealed that the diversity within clade I (i.e., *E. latifolium* inhabiting the headwater riches of each river) was greater than that of clade II (i.e., *E. l-nigrus* inhabiting the wider Shinano-gawa river basin area). Also, it was revealed that the haplotype diversity (h) and the nucleotide diversity (π) within full study scales (basin scales) of clade I (i.e., *E. latifolium*) was greater than those in clade II (i.e., *E. l-nigrus*) (Table 2). In particular, with respect to the nucleotide diversity, it was revealed that the diversification in clade I (i.e., *E. latifolium*) is greater than those in clade II (i.e., *E. l-nigrus*) for any scale of river. Although no significant differences were shown by AMOVA analyses in any scale of river (Table 3), the trend of the relationship between genetic difference and habitat segregation was established; that is, the genetic diversity in clade I (i.e., *E. latifolium*) is greater than those in clade II (i.e., *E. l-nigrus*) for any scale of river. Low gene flow within headwater species may be a common feature observable in many aquatic insects due to physical barrier effect of mountain-valleys (Hughes 2007).

In the last decade, there have been an increasing number of cases of genetic

analysis, to elucidate the population structure of aquatic insects (*e.g.*, Hughes et al. 1999, 2003a, b; Monaghan et al. 2001, 2002; Wilcock et al. 2003; Smith et al. 2006; Hughes 2007; Mérria and Hughes 2008; McCulloch et al. 2009).

Traditionally, ecological research of river systems has often been based primarily upon macro-benthos in the nymphal stages of insect species. As a result, it is inherently difficult to reliably differentiate discrete species in many cases. However, in this study, we were able to successfully overcome the limitation by reliably differentiating heptageniid mayflies, *via* the 'DNA Bar-coding' method: An identification system based on DNA sequence comparison using the mitochondrial DNA (COI region) data sequence (*e.g.*, the "International Barcode of Life" project or "Global Bioidentification System"; Hebert 2003). We have shown that by the reliable identification of species such as *E. latifolium* and *E. l-nigrus* using genetic analysis that we can overcome the earlier limitations that restricted the ability to correctly differentiate species. We conclude that the effective use of genetic analysis of aquatic insects will be one of the useful approaches to elucidating population structures and also river ecosystems.

ACKNOWLEDGMENTS

We acknowledge the valuable suggestions and support of Professors H. Nakamura (Shinshu University), K. Tanida (Osaka Prefecture University), Y. Takemon (Kyoto University), Y. Shimatani (Kyushu University), and Mr. K. Inada (Himeji High School). We express our thanks to the Chikuma River Office (Hokuriku Regional Development Bureau, Ministry of Land Infrastructure and Transport) and the Chikuma River Ecology Research Group, for their cooperation with research at the study site. We are also indebted to Mr/s Y. Tanaka (Shinshu University) for their cooperation in field research.

References

Bae YJ (1997) A historical rview of Ephemeroptera systematics in Northeast Asia. In: Landolt P, Satrori M (eds) Ephemeroptera & Plecoptera: Biology-ecology-systematics. Mauron, Tinguely & Lachat SA, Moncor,

Fribourg, pp 405-417

Bae YJ, Yoon IB (1997) A revised catalogue of the Ephemeroptera of Korea. Entomol Res Bull 23:43-53

Bae YJ, Yoon IB, Chun DJ (1994) A catalogue of the Ephemeroptera of Korea. Entomol Res Bull 20:31-50

Bae YJ, Lee JE, Yoon IB (2000) Northeast Asian Ephemeroptera in Imanishi's 1940 report. Entomol Sci 3:391-397

Excoffier L, Larval G, Schneider S (2005) Arlequin ver 3.0: An integrated software package for population genetcs data analysis. Evol Bioinform Online 1:47-50

Felsenstein J (1995) Confidence limits on phylogenies: An approach using the bootstrap. Evol 39:783-791

Folmer O, Black M, Hoeh W, Lutz R, Vrijienchoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294-299

Hebert PDN, Cywinska A, Ball SL, DeWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci 270:313-321

Hou Z, Fu J, Li S (2007) A molecular phylogeny of the genus *Gammarus* (Crustacea: Amphipoda) based on mitochondria and nuclear gene sequences. Mol Phyl Evol 45:596-611

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755

Hughes JM, Mather PB, Sheldon AL, Allendorf FW (1999) Genetic structure of the stonefly, *Yoraperla brevis*, populations: the extent of gene flow among adjacent montane sterams. Freshw Biol 41:63-72

Hughes JM, Hillyer M, Bunn SE (2003a) Small-scale patterns of genetic variation in the mayfly *Bungona narilla* (Ephemeroptera: Baetidae) in rainforest streams, south-east Queensland. Freshw Biol 48:709-717

Hughes JM, Mather PB, Hillyer MJ, Cleary C, Peckarsky B (2003b) Genetic structure in a montane mayfly *Baetis bicaudatus* (Ephemeroptera: Baetidae), from the Rocky Mountains, Colorado. Freshwat Biol 48:2149-2162

Hughes JM (2007) Constrains on recovery: using molecular methods to study connectivity of aquatic biota in rivers and streams. Freshw Biol 52:616-631

Inada K (2007) Distribution of Epeorus l-nigrus and E. latifolium at the Ibokawa

river, Hyogo, Japan. Hyogo Freshw Biol 59:111-112

Ishiwata S, Takemon Y (2005) Ephemeroptera. In: Kawai T, Tanida K (eds) Aquatic insects of Japan: Manual with keys and illustrations. Tokai University Press, Tokyo, pp 31-128

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111-120

Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the *Isthmus* of Panama. Proc R Soc Lond B 265:2257-2263

Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9:299-306

Kluge N (2004) The phylogenetic system of Ephemeroptra. Kluwer Academic Publishers, Dordrecht

McCulloach GA, Wallis GP, Waters JM (2009) Do insects lose flight before they lose their wing? Population genetic structure in subalpine stoneflies. Mol Ecol 18:4073-4087

Mérria C, Hughes JM (2008) Cyclic habitat displacements during Pleistocene glaciations have induced independent evolution of *Tasimia palpata* populations (Trichoptera: Tasimiidae) in isolated subtropical rain forest patches. J Biogeogr 35:1717-1737

Monaghan MT, Spaak P, Robinson CT, Ward JV (2001) Genetic deifferentiation of *Baetis alpinus* Pictet (Ephemeroptera: Baetidae) in fragmented alpine streaams. Heredity 86:395-403

Monaghan MT, Spaak P, Robinson CT, Ward JV (2002) Population genetic structure of 3 alpine stream insects: influences of gene flow, demographics, and habitat fragmentation. J N Am Benthol Soc 21:114-131

Queck SP, Davis SJ, Itino T, Pierce NE (2004) Codiversification in an ant-plant mutualism: Stem texture and the evolution of host use in *Crematogaster* (Formicidae: Myrmicinae) inhabitants of *Macaranga* (Euphorbiaceae). Evolution 58:554-570

Rozas J, Sanchez-Delbarrio J, Messeguer X, Rogas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformations

19:2496-2497

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Smith PJ, Mcveagh M, Collier AK (2006) Genetic diversity and historical population structure in the New Zealand mayfly *Acanthophlebia cruentata*. Freshw Biol 51:12-24

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acid Res 22:4673-4680

Wilcock HR, Nichols RA, Hildrew AG (2003) Genetic population structure and neighborhood population size estimates of the caddisfly *Plectrocnemia conspersa*. Freshw Biol 48:1813-1824

Yoon IB, Bae YJ (1984) The classification of Heptageniidae (Ephemeroptera) in Korea. Entomol Res Bull 10:1-34

Figure Legends

Fig. 1 Location of the Shinano-gawa River basin, and the 30 study sites (the Chikuma-gawa River main stream, C1-11; the Sai-gawa River, S1-4; the Azusa-gawa River, A1-4; the Narai-gawa River, N1-6; the Takase-gawa River, T1-5).

Fig. 2 Proportions of *Epeorus latifolium* and/or *Epeorus l-nigrus* mayflies of all the collected heptageniid mayflies, at each study site throughout the year (four seasons).

Fig. 3 Genetic relationship between *Epeorus latifolium* and *Epeorus l-nigrus* mayflies based on 521bp mitochondrial COI sequences. 98 nymphal specimens from 30 populations of *Epeorus latifolium* and/or *Epeorus l-nigrus* were used with 6 male specimens of *E. latifolium* and 3 male specimens of *E. l-nigrus*. All of the GenBank accession numbers are listed in Table 1 (the accession numbers of male adults and the outgroup are written in the text). The NJ tree was constructed based on genetic distances calulated by Kimura's two-parameter modes using the species *Epeorus aesculus* as an outgroup. The scale bar indicates substitutions per site. The topologies presented by MP and Bayesian trees are essentially identical to those presented by the NJ tree. NJ (left) and MP (center) bootstrap values, and Bayesian posterior probabilities (right) are specified, when they exceed 50%.

Fig. 4 Re-mapping of the collection sites of *Epeorus latifolium* and *Epeorus l-nigrus*. The specimens included in 'clade I' in Fig. 3 are shown as solid circles (●; these being the species *E.latifolium*), and the specimens included in 'clade II' in Fig. 3 are shown as open circles (○; these being the species *E. l-nigrus*). The sites including both members clade I and clade II (*i.e.*, *Epeorus latifolium* and *Epeorus l-nigrus*) are shown as meshed marks (see explanatory notes in the figure).

Table 1. Study site information and the information of specimens using genetic analyses

No. of study site	Latitude (N)	Longitude (E)	Altitude	Examined specimens No. for DNA analysis (sampling month)	Accession No.
Chikuma-gawa River					
C1	35° 56' 17"	138° 42' 45"	1,490m	2 (1n: Jul 08; 1n: Mar 09)	AB538324-25
C2	35° 56' 94"	138° 42' 36"	1,470m	3 (3n: Jul 08)	AB538326-28
C3	35° 57' 68"	138° 42' 85"	1,460m	1 (1n: Jul 08)	AB538329
C4	35° 57' 76"	138° 42' 92"	1,410m	-	
C5	35° 57' 44"	138° 40' 22"	1,305m	1 (1n: Jul 08)	AB538341
C6	35° 58' 05"	138° 37' 92"	1,245m	2 (2n: Jul 08)	AB538342-43
C7	35° 58' 47"	138° 34' 48"	1,180m	13 (11n: Jul 08; 2n: Mar 09)	AB538344-47, AB550562-70
C8	36° 14' 66"	138° 27' 59"	665m	10 (1n: Jul 08; 9n: Mar 09)	AB538348-51, AB550571-76
C9	36° 22' 10"	138° 17' 76"	470m	2 (1n: Jul 08; 1n: Mar 09)	AB538352-53
C10	36° 32' 02"	138° 06' 71"	360m	1 (1n: Mar 09)	AB538354
C11	36° 42' 76"	138° 17' 22"	325m	8 (8n: Mar 09)	AB538355-58, AB550577-80
Narai-gawa River					
N1	35° 52' 72"	137° 50' 37"	1,190m	-	
N2	35° 52' 30"	137° 49' 66"	1,200m	-	
N3	35° 57' 15"	137° 48' 24"	970m	1 (1n: Jul 08)	AB538330
N4	35° 58' 93"	137° 49' 39"	910m	7 (1n: Jul 08; 6m: Sep 09)	AB538331, AB538373-75, AB538379-81
N5	36° 02' 36"	137° 53' 85"	795m	1 (1n: Jul 08)	AB538359
N6	36° 09' 76"	137° 56' 85"	640m	6 (6n: Mar 09)	AB538360-61, AB550605-08
Azusa-gawa River					
A1	36° 15' 07"	137° 39' 11"	1,530m	1 (1n: Jul 08)	AB538332
A2	36° 09' 72"	137° 39' 41"	1,000m	3 (1n: Jul 08; 2n: Apr 09)	AB538333-35
A3	36° 10' 43"	137° 47' 39"	725m	5 (1n: Jul 08; 1n: Mar 09; 3m Aug 09)	AB538336-37, AB538376-78
A4	36° 14' 22"	137° 54' 50"	610m	12 (1n: Jul 08; 11n: Mar 09)	AB538362-64, AB550560-61, AB550598-604
Takase-gawa River					
T1	36° 29' 28"	137° 44' 99"	910m	1 (1n: Jul 08)	AB538338
T2	36° 29' 71"	137° 44' 48"	910m	1 (1n: Jul 08)	AB538339
T3	36° 30' 65"	137° 47' 78"	800m	1 (1n: Jul 08)	AB538340
T4	36° 29' 16"	137° 51' 97"	750m	-	
T5	36° 23' 07"	137° 52' 36"	570m	1 (1n: Jul 08)	AB538365
Sai-gawa River					
S1	36° 17' 13"	137° 56' 25"	540m	11 (1n: Jul 08; 10n: Mar 09)	AB538366, AB550581-90
S2	36° 22' 76"	137° 55' 15"	510m	1 (1n: Jul 08)	AB538367
S3	36° 32' 40"	137° 58' 43"	435m	10 (1n: Jul 08; 9n: Apr 09)	AB538368-70, AB550591-97
S4	36° 37' 97"	138° 07' 20"	370m	2 (1n: Jul 08; 1n: Apr 09)	AB538371-72
30 sites				107 specimens	

m: male adult(s)
n: nymph(s)

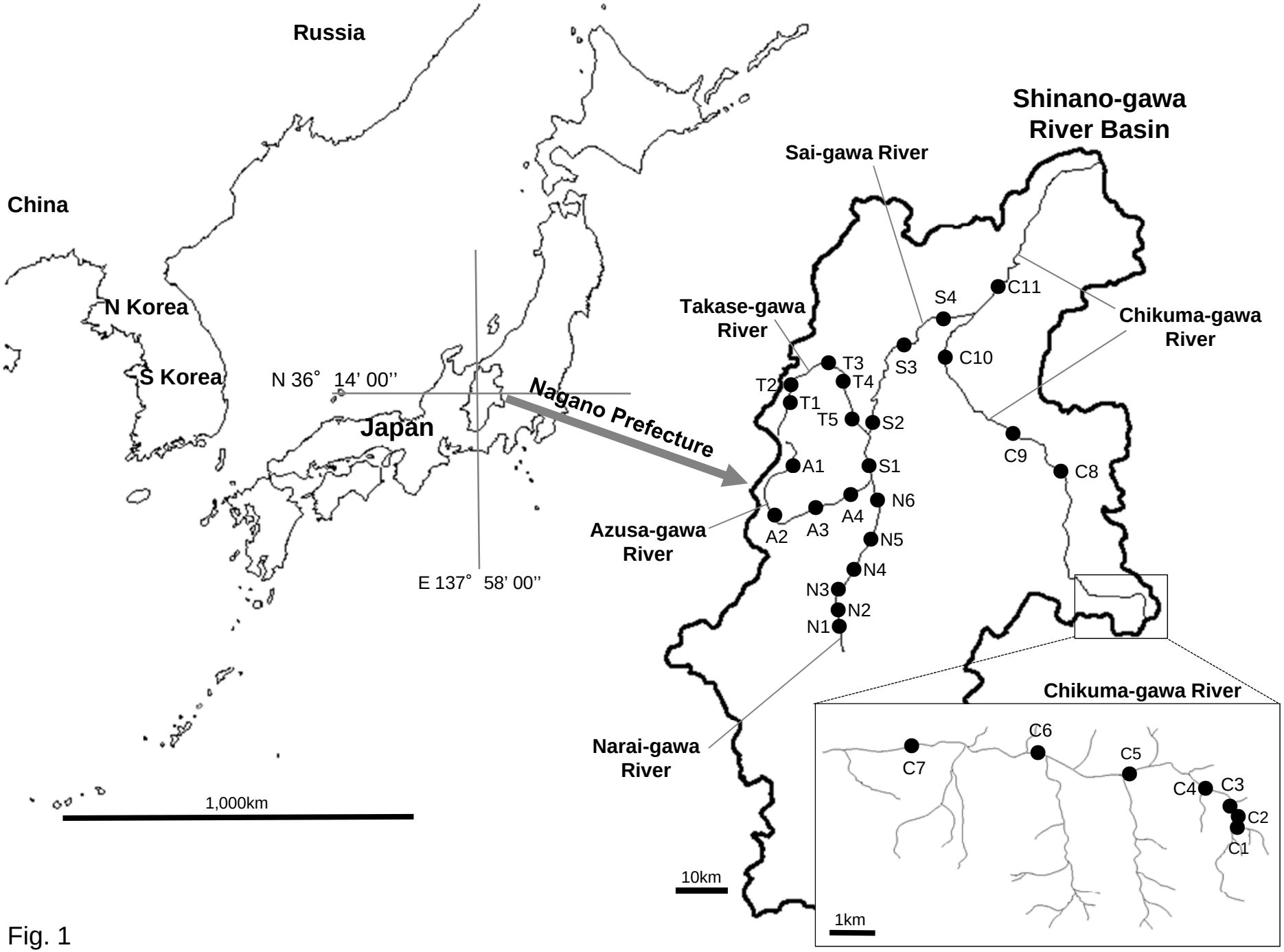
Russia
China
N Korea
S Korea
Japan
N 36° 14' 00''
E 137° 58' 00''
Nagano Prefecture
1,000km
Shinano-gawa
River Basin
Sai-gawa River
Takase-gawa
River
Chikuma-gawa
River
Azusa-gawa
River
Narai-gawa
River
S4
C11
S3
C10
T3
T4
T2
T1
T5
S2
C9
C8
A1
S1
N6
A4
A3
A2
N5
N4
N3
N2
N1
10km
Chikuma-gawa River
C6
C7
C5
C4
C3
C2
C1
1km

Fig. 1

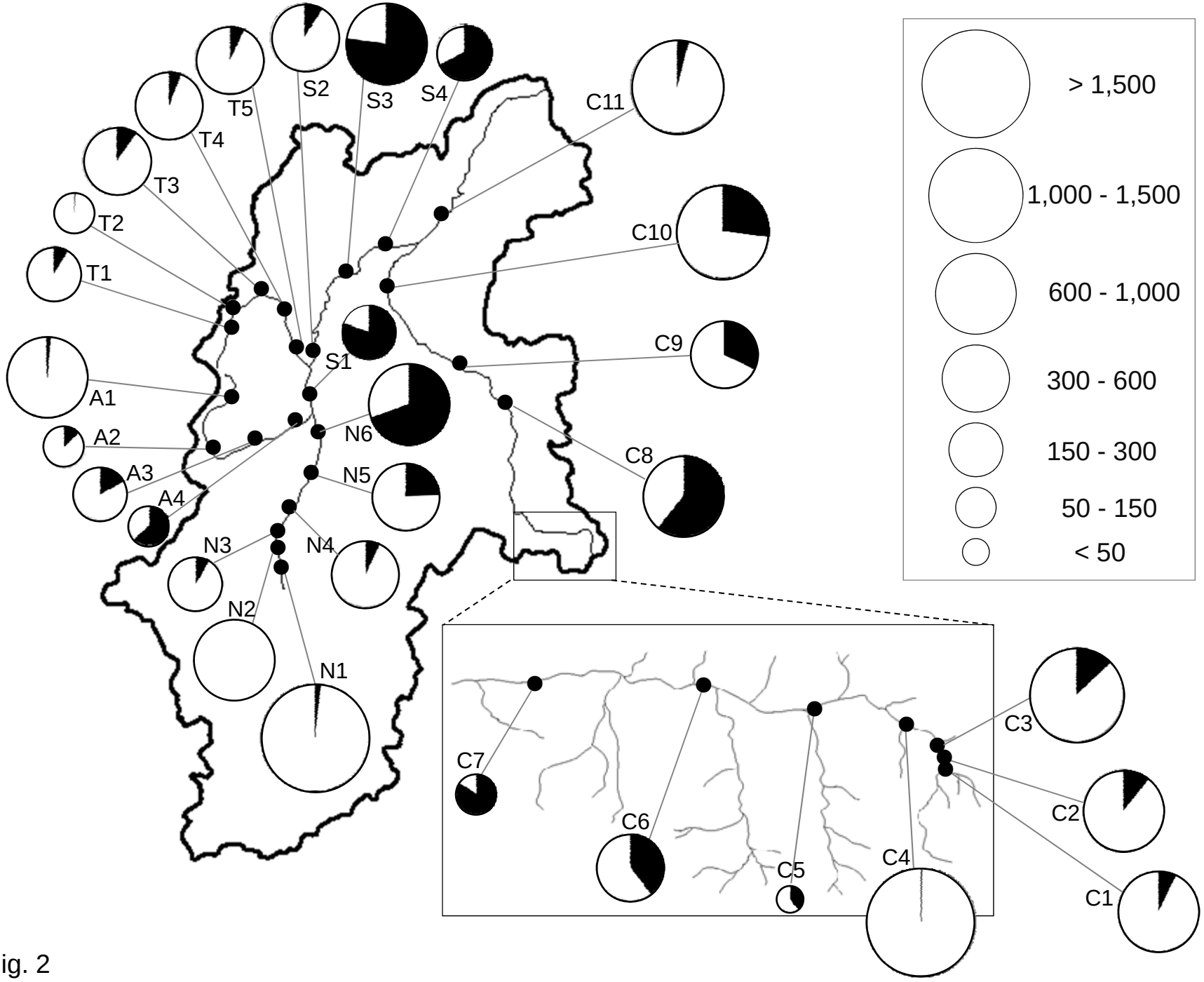
S2
S3
S4
T5
T4
T3
T2
T1
C11
C10
C9
C8
S1
A1
A2
A3
A4
N6
N5
N3
N4
N2
N1
> 1,500
1,000 - 1,500
600 - 1,000
300 - 600
150 - 300
50 - 150
< 50
C7
C6
C5
C4
C3
C2
C1

Fig. 2

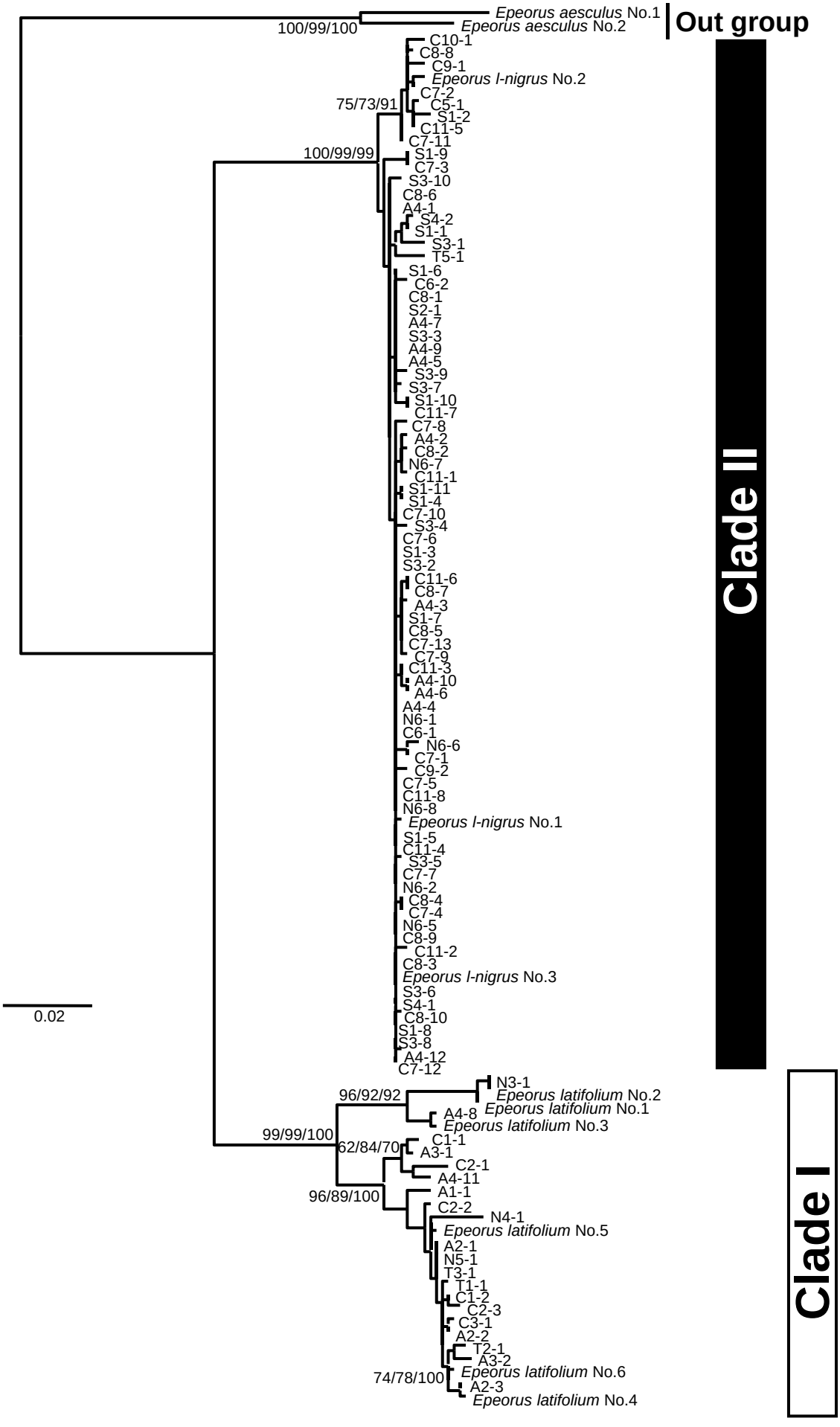
Epeorus aesculus No.1
Epeorus aesculus No.2
100/99/100
Out group
C10-1
C8-8
C9-1
Epeorus l-nigrus No.2
C7-2
75/73/91
C5-1
S1-2
C11-5
C7-11
100/99/99
S1-9
C7-3
S3-10
C8-6
A4-1
S4-2
S1-1
S3-1
T5-1
S1-6
C6-2
C8-1
S2-1
A4-7
S3-3
A4-9
A4-5
S3-9
S3-7
S1-10
C11-7
C7-8
A4-2
C8-2
N6-7
C11-1
S1-11
S1-4
C7-10
S3-4
C7-6
S1-3
S3-2
C11-6
C8-7
A4-3
S1-7
C8-5
C7-13
C7-9
C11-3
A4-10
A4-6
A4-4
N6-1
C6-1
N6-6
C7-1
C9-2
C7-5
C11-8
N6-8
Epeorus l-nigrus No.1
S1-5
C11-4
S3-5
C7-7
N6-2
C8-4
C7-4
N6-5
C8-9
C11-2
C8-3
Epeorus l-nigrus No.3
S3-6
S4-1
C8-10
S1-8
S3-8
A4-12
C7-12
Clade II
0.02
N3-1
96/92/92
Epeorus latifolium No.2
Epeorus latifolium No.1
A4-8
Epeorus latifolium No.3
99/99/100
C1-1
62/84/70
A3-1
C2-1
A4-11
96/89/100
A1-1
C2-2
N4-1
Epeorus latifolium No.5
A2-1
N5-1
T3-1
T1-1
C1-2
C2-3
C3-1
A2-2
T2-1
A3-2
74/78/100
Epeorus latifolium No.6
A2-3
Epeorus latifolium No.4
Clade I

Fig. 3

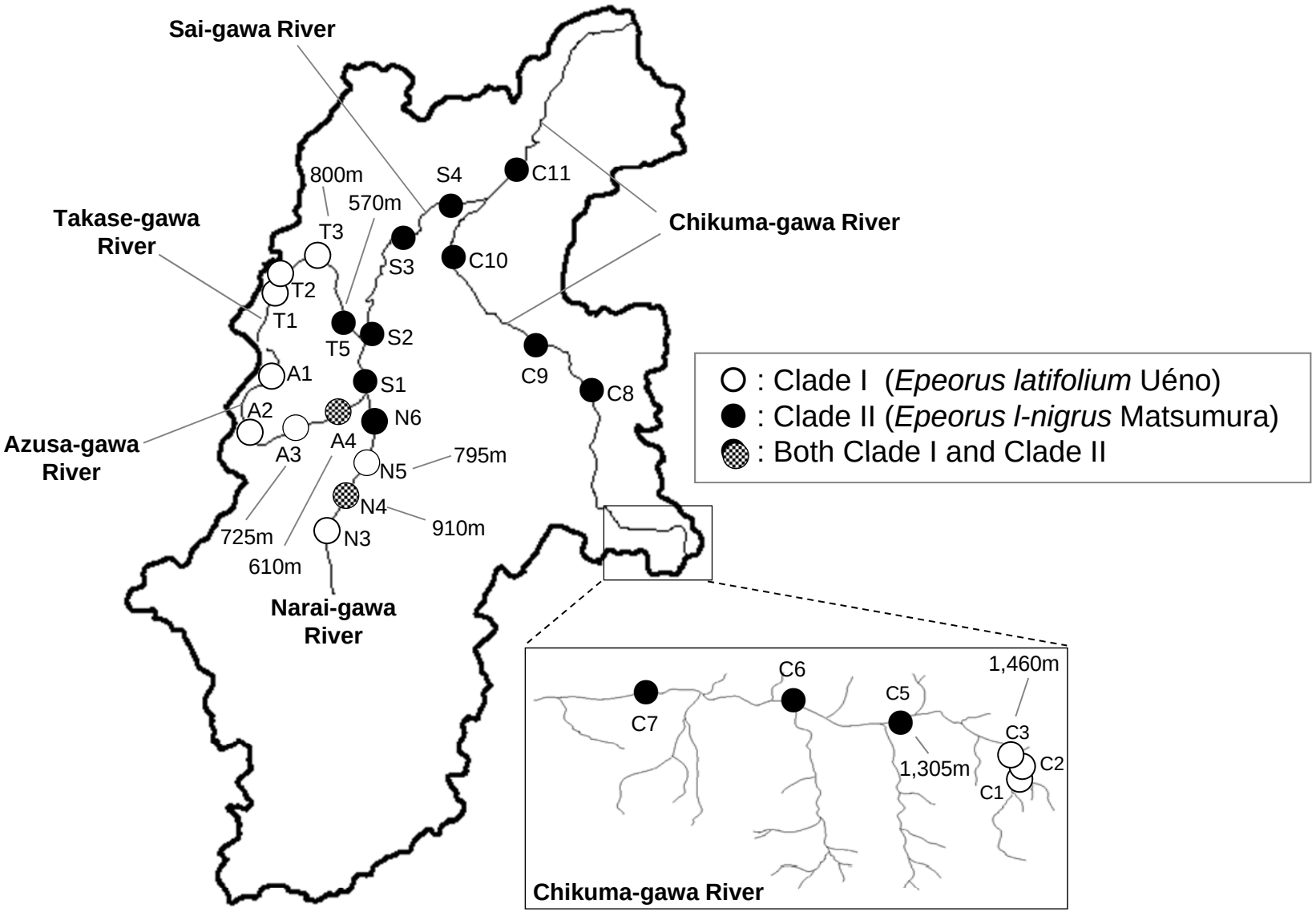
Sai-gawa River
Takase-gawa River
Azusa-gawa River
Narai-gawa River
Chikuma-gawa River
800m
570m
725m
610m
910m
795m
1,460m
1,305m
T1
T2
T3
T5
S1
S2
S3
S4
A1
A2
A3
A4
N3
N4
N5
N6
C1
C2
C3
C5
C6
C7
C8
C9
C10
C11
○ : Clade I (*Epeorus latifolium* Uéno)
● : Clade II (*Epeorus l-nigrus* Matsumura)
: Both Clade I and Clade II
Chikuma-gawa River

Fig. 4