1   **Molecular phylogeny and diversification timing of the Nemouridae family (Insecta,**

2   **Plecoptera) in the Japanese Archipelago**

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13   Short Title: Molecular phylogeny and diversification timing of Nemouridae

14

15   **Abstract**

16   The generation of the high species diversity of insects in Japan was profoundly influenced by the

17   formation of the Japanese Archipelago. We explored the species diversification and biogeographical

18   history of the Nemouridae family in the Japanese Archipelago using mitochondrial DNA and nuclear

19   DNA markers. We collected 49 species among four genera: *Indonemoura*, *Protonemura*,

20   *Amphinemura* and *Nemoura* in Japan, China, South Korea and North America. We estimated their

21   divergence times—based on three molecular clock node calibrations—using Bayesian phylogeography

22   approaches. Our results suggested that Japanese Archipelago formation events resulted in

23   diversification events in the middle of the Cretaceous (<120 Ma), speciation in the Paleogene (<50 Ma)

24   and intra-species diversification segregated into eastern and western Japan of the Fossa Magna region

25   at late Neogene (20 Ma). The *Indonemoura* samples were genetically separated into two clades—that

26   of Mainland China and that of Japan. The Japanese clade clustered with the Nemouridae species from

27   North America, suggesting the possibility of a colonisation event prior to the formation of the Japanese

28   Archipelago. We believe that our results enhanced the understanding both of the origin of the species

29   and of local species distribution in the Japanese Archipelago.

30

31   Keywords: stoneflies, Japan, divergence time, molecular phylogeny, speciation

32

Introduction

33

34   The East Asian region—and in particular, the Japanese Archipelago—is considered to have

35   high insect biodiversity [1], [2]. The high degree of Japanese insect biodiversity is a result of several

36   mechanisms—in particular, the complex geological history. The Japanese Archipelago originated in

37   the middle of the Miocene [3] as an independent formation of eastern and western Japanese

38   landmasses. Extensive geographical changes and large-scale climatic changes throughout the islands

39   facilitated the subsequent connection and disconnection of Japanese landmasses from the Eurasian

40   continent, and the formation of tectonic lines (as the median tectonic line, MTL; and the Itoigawa-

41   Shizuoka tectonic line, ISLT) [3], [4], [5]. These geological events—allowing for the colonisation of

42   insects from the continent and their subsequent diversification as endemic lineages (i.e. new

43   species)—contributed substantially to the high diversity of insects in Japan [2].

44   The process of species diversification has been intensively explored through

45   phylogeographical approaches [6], [7]. These approaches have allowed for the observation of the

46   historical process responsible for the current geographical distribution of individuals [6]. Molecular

47   approaches to phylogeographic studies, using specific genes—such as mitochondrial DNA (mtDNA) or

48   nuclear DNA (nDNA)—allow for a better understanding of species diversity by resolving complex

49   taxonomic groups of species (for instance, cryptic species and species groups) [7]. Molecular

50   phylogeography has provided valuable insights into the historical process of Japanese Archipelago

51   formation underlying insect diversification. Previous studies identified genetic differentiation within

52   species between the Japanese landmasses and the Eurasian continent (for instance, the mayflies

53   *Isonychia japonica* [8]; caddisflies *Palaeagapetus* spp. [9]; and beetles *Ohomopterus* spp. [10] and the

54   Carabina subtribe [11]). Dispersal events via land bridges (islands between continents) from the

55   Eurasian continent to the Japanese Archipelago (of, for instance, the orthopteran *Locusta migratoria*,

56   [12]; mayflies *Ephron* spp., [13]) or, in reverse, from the Japanese Archipelago to the Eurasian

57   continent (of, for instance, water bugs *Appasus* spp., [14]) were additionally identified before, during

58   and after the formation of the Japan Archipelago.

59   Aquatic insects have advantages in the studies of phylogeography, as their specialised

60   ecological requirements and habitat range make aquatic insect species susceptible to geological

61   changes. Among the Plecoptera order [15], the family Nemouridae is one of the largest and most

62   dominant aquatic insect groups. The family comprises 20 genera and more than 400 species

63   distributed throughout the Northern Hemisphere and across the equator in the Sunda Archipelago

64   [16]. Several genera of the Nemouridae family have distinct disjunctions in their distribution [15]. For

65   example, *Ostrocerca, Prostoia* and *Soyedina* were found in both the extreme western and the extreme

66   eastern regions of North America, but they were absent in the central area [17], [18]. Similar

67   disjunctive distributions were also observed among *Protonemura*, *Indonemoura*, *Sphaeronemoura*

68   and *Illiesonemoura* in the Palaearctic region [19], the western and eastern Himalayan ranges [20] and

69   North and South India [15]. *Podmosta* and *Zapada* are two interesting cases distributed across the

70   Nearctic region and East Asia [21], [22]. Previous studies have suggested that their current habitat

71   distribution could be associated with mountain formation and land bridges. In Japan, the Nemouridae

72   family is widely distributed with four genera [23] —*Indonemoura*; *Protonemura*; *Amphinemura*; and

73   *Nemoura.* To date, 30 *Nemoura* species, 17 *Amphinemura* species, 12 *Protonemura* species and 1

74   *Indonemoura* species have been reported in Japan [16]. However, their evolutionary history in the

75   Japanese Archipelago remains unknown.

76   We studied the molecular phylogeny of the aquatic insect Nemouridae (Plecoptera) in the

77   Japanese Archipelago with comprehensive genera-level sampling using mitochondrial cytochrome c

78   oxidase 1 (*cox1*) and nuclear histone 3 (*H3*) markers. We hypothesised that the Nemouridae family

79   diversification could be linked to the geological formation of the Japanese Archipelago. Therefore, we

80   estimated the phylogenetic relationships among Nemouridae species and genera with reference to

81   their historical biogeography. We focused on geographic events of Japanese Archipelago formation

82   and their influence on the divergence time among the genera and species using a combination of fossil

83   records and the Archipelago formation history. Furthermore, to estimate the historical process of the

84   phylogeography of Nemouridae in Japan, we compared the phylogenetic relationships among the

85   specimens from South Korea, China and North America, that are assumed to be the potential sources

86   of Japanese Nemouridae because of the geological formation history of the Japanese Archipelago.

87

88

89   **Material and methods**

90

91   **Study sites and sample collection**

92   Our sampling sites in Japan comprised 32 sampling sites on Hokkaido Island, 83 on Honshu

93   Island and 27 on Shikoku Island. None of the Nemouridae species was found on Hokkaido Island during

94   sampling. All species reported from Hokkaido are known to occur on either Honshu or Shikoku Islands.

95   Herein, we only reported on the sampling sites wherein specimens where found. We collected 20, 7,

96   8 and 1 species of the genera *Nemoura*, *Amphinemura*, *Protonemura* and *Indonemoura*, respectively,

97   on 110 sampling sites in Japan (Fig 1, S1 Table). Additionally, 14 species distributed from 8 sampling

98   sites of Mainland China and 2 of South Korea (S1 Table, Fig 2) and 100 specimens of the three species

99   *Zapada columbiana*, *Z. cinctipes* and *Podmosta delicatula* (subfamily Nemourinae) collected from 15

100   sampling sites of North America (western United States of America and Alaska) were included in our

101 analysis. We added these samples from outside of Japan because of their geographical proximity to

102 the Japanese Archipelago and their geological formation histories.

103 We collected adult insects using hand nets around riversides. We stored samples in 80%

104 ethanol in the field, and replaced the ethanol with fresh 99.5% ethanol after morphological

105 identification. We identified individuals according to the taxonomical keys of [21], [23], [24], [25], [26],

106 [27], [28], [29], [30], [31] and [32]. Undescribed species resulted in our studied were based on our

107 taxonomic expertise and inconclusive taxonomic keys.

108

109 **DNA extraction, amplification and sequencing**

110 We genetically analysed a total of 289 individuals, out of which 189 were from East Asia

111 (males, 97; females, 92) and 100 were from North America (males, 92; females, 8). We extracted

112 genomic DNA individually using DNeasy tissue kits (Qiagen GmbH, Hilden, Germany), following the

113 manufacturer's instructions. We amplified a 658-bp fragment of mtDNA *cox1* using LCO-1490 and

114 HCO-2198 primers [33] with an annealing temperature of 38°C and 40 PCR cycles. Further, we

115 amplified a 328-bp fragment of nDNA marker histone 3 (*H3*) using the universal primers H3F and H3R

116 [34] with an annealing temperature of 58°C and 40 PCR cycles. We purified the PCR products using

117 the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and sequenced them in both

118 directions using the same primers as mentioned above. *Cox1* and *H3* sequences were sequenced by

119 Eurofins Operon (Tokyo, Japan). All sequence data reported here have been deposited in GenBank.

120

121 **Sequence analysis**

122 We assembled and edited forward and reverse sequences using CodonCode Aligner v 3.5

123 (Codon Code Corporation, Dedham, USA). All sequences were aligned using ClustalW

124 (https://www.genome.jp/tools-bin/clustalw) [35]. We calculated the genetic diversity by the number

125 of polymorphic sites, number of haplotypes and both mean nucleotide substitution rate (i.e individuals

126 within species) and pairwise nucleotide substitution rate (i.e between species), with the Kimura 2-

127 parameter model. We performed all analyses using DnaSp v5.10 [36]. All analyses were performed for

128 *cox1* and h3 separately.

129 All sequences of the mtDNA and nDNA markers were compared with the NCBI nucleotide

130 database using blastn queries (http://blast.ncbi.nlm.nih.gov) to corroborate species identification

131 (DNA barcoding, similarity > 98%) and to discard possible sequence errors.

132

133 **DNA species delimitation**

134   To corroborate the morphological species identification match with our molecular data, we

135   implemented a DNA species delimitation analysis. Putative DNA species were delineated using the

136   General Mixed Yule Coalescent model (GMYC; [37]). An ultrametric gene tree of *cox1* gene was

137   constructed using BEST v1.8.3 [38], and the GMYC analysis was performed using the splits package

138   [39] in R ver. 3.3 (R Core Team).

139

140   **Molecular clock analysis**

141   We estimated the evolutionary history of the family in the Japanese Archipelago according to

142   the timing of the divergence of the lineages. For this estimation, we implemented a Bayesian

143   phylogenetic analysis in combination with a molecular clock analysis using BEAST v.2.4.4 [40] with

144   *Zwicknia bifrons* (Capniidae) as a outgroup for *cox1* and *H3* (own sequences) separately. This outgroup

145   was selected owing to their close phylogenetic relationship with Nemouridae [41], [42]. To observed

146   the divergence time, we adopted a relax clock model [43] following a log normal distribution, and

147   calibrated the phylogenetic tree nodes using three types of molecular clock analysis. The first

148   calibration was based on fossil records of the Nemouridae family [44]. We calibrated the nodes at 180

149   million years ago (Ma) and adjusted the parameters with a standard deviation of 20 Ma, as suggested

150   in previous study [45] for a 95% highest posterior density (HPD). For this analysis, we implemented a

151   fossilised birth death model [46] for tree prior parameter. The second calibration was based on the

152   Japanese Archipelago formation events dated from 15 to 30 Ma [3]. We applied several calibrations

153   from 15, 20, 25 and 30 Ma at all nodes representing taxonomic species. All calibrations were adjusted

154   to 5 Ma as a standard deviation for a 95% HPD and a fossilised birth death model [46] for tree prior

155   parameter. Lastly, the third calibration was the time to the most recent common ancestor (TMRCA)

156   to observe species diversification patterns based on the mean substitution rate of *cox1*. Using a Yule

157   model tree prior parameter [47], we applied the substitution rate for insect *cox1* of 1.5% [48] and

158   3.54% [49] per million years for a 95% HPD.

159   For all branch age calibrations (namely, fossil, biogeographic and mtDNA substitution rate),

160   we performed MCMC for 50 million generations, and log dating trees (BEAST parameters) for every

161   5000 generations. We tested the output files for convergence after removing a 10% burn-in by

162   examining the effective sampling size using Tracer v1.5 [50]. We pooled the four resulting output trees

163   from biogeographical calibration analysis into a single tree. We then pooled the resulting single tree

164   from biogeographical branch calibration and the single tree from fossil calibration analyses into a

165   single tree. We performed all pooling analyses using Log Combiner v1.6.1 (BEAST package)

166   summarised with Tree Annotator (BEAST package) and visualised using FigTree v1.3.1 [51]. We

167   performed the analyses for *cox1* and *H3* separately. The incongruence length difference test (ILD) [52]

168   was conducted to test the congruence of tree topologies between *cox1* and *H3* using Tree Analysis

169   Technology (TNT) [53]. ILD test revealed no significant differences in terms of the Bayesian tree

170   topologies between *cox1* and *H3* (P = 0.8); therefore, both markers were polled into a single tree for

171   further analysis.

172

173   **Phylogenetic analysis between Nemoura from Japan and North America**

174   To observe the phylogenetic relationship between Nemouridae from Japan and North

175   America (*Zapada columbiana*, *Z. cinctipes* and *Podmosta delicatula*), we analysed the maximum

176   likelihood (ML) phylogenetic trees of *cox1* and *H3* separately using PhyML 3.1 [54]. The General time-

177   Reversible (GTR) model and gamma distribution were selected for both markers (*cox1* and H3) based

178   on separate test performed with jModel Test v.3 [55] and using *Zwicknia bifrons* (Capniidae) as an

179   outgroup as described above. The trees were bootstrapped using 10,000 replications.

180

181   **Results**

182

183   **Genetic diversity and DNA phylogeny**

184   For studying the phylogeny of Nemouridae in the Japanese Archipelago, we analysed two

185   molecular markers. *Cox1* sequences were of 658 bp length, with 247 polymorphic sites, 237

186   parsimony-informative sites, 10 singletons and a mean nucleotide substitution rate of 0.151. *H3*

187   sequences were of 328 bp length, with 67 polymorphic sites, 54 parsimony-informative sites, 13

188   singletons and a mean nucleotide substitution rate of 0.051. No gaps were detected for either *cox1* or

189   *H3* sequences (S1, S2 Fig). In total, for *cox1* and *H3*, we identified 128 and 68 haplotypes, respectively.

190   The GMYC model of *cox1* delimited 61 putative DNA-species (S1 Table). These results agreed

191   with our 34 morphologically identified and 15 undescribed (five species of *Protonemura*, seven of

192   *Nemoura*, one of *Indonemoura* and two of *Amphinemura*) species. Eight species (*I. nohirae*, *A.*

193   *decemseta*, *A. zonata*, *A. longispina*, *A. megaloba*, *N. uenoi*, *N. chinonis* and *N. cf. cercispinosa*) showed

194   two putative DNA-species. While *A. decemseta* showed multiple putative DNA-species (three putative

195   DNA-species), *N. sanbena* and *P. kohnoae* showed two putative DNA-species in the same sampling site

196   suggesting the presence of cryptic species. The congruence of H3 phylogenetic groups provided

197   confirmation of DNA-based groups detected by GMYC.

198   We observed the genetic diversity of the species per island (Table 1). Honshu had the highest

199   number of species (26 species), haplotype richness (63) and mean nucleotide substitution rate

200   (average 0.027). Five species were found throughout the three Japanese islands (Honshu, Shikoku and

201   Kyushu), i.e. *A. decemseta*, *A. zonata*, *N. cf. cercispinosa*, *N. chinonis* and *I. nohirae*, with a mean

202 nucleotide substitution rate ranging from 0.011 to 0.126 and a total of 23 haplotypes. *N. sanbena*

203 haplotypes were observed in two different branches in the phylogenetic tree, both within *N. cf.*

204 *cercispinosa* and as an isolated branch.

205

Divergence dates

207   The Bayesian phylogenetic trees for *cox1* and *H3* showed tree topology similarity (ILD test, P

208 = 0.8). Three clades corresponded to the three families—*Protonemura*, *Amphinemura* and

209 *Nemoura*—whereas *Indonemoura* was divided into two clades—the Mainland China clade, clustered

210 with *Protonemura*, and the Japanese clade (Fig 2).

211   The evolutionary divergence between the Nemouridae and Capniidae families was settled at

212 180 Ma, with a 95% HPD interval of 160 to 198 Ma, in the Jurassic geological period (Fig 2, S3 and S4

213 Fig). Genus-level diversifications within Nemouridae occurred in the early and middle Cretaceous.

214 *Indonemoura* from Japan at 119.0 Ma (95% HPD, 125.8 to 100.2 Ma), *Indonemoura* from Mainland

215 China at 112.0 Ma (95% HPD, 90.2 to 115.0 Ma), *Protonemura* at 112.7 Ma (95% HPD, 98.0 to 121.3

216 Ma), *Nemoura* at 107.0 Ma (95% HPD, 98.8 to 110.1 Ma) and *Amphinemura* at 80.0 Ma (95% HPD,

217 75.1 to 92.0 Ma). The speciation process occurred between 25 Ma (early Paleogene) and 90 Ma (late

218 Crustaceous). Out of 35 events of speciation (i.e. nodes), 16 (45%) occurred during late Crustaceous

219 and 19 (54%) occurred during early Paleogene, broadly overlapping with the formation time of the

220 Japanese Archipelago (15 to 30 Ma). We observed intra-species diversification in *I. nohirae*, *A.*

221 *decemseta*, *A. zonata*, *A. longispina*, *A. megaloba*, *N. chinonis*, *N. uenoi* and *N. cf. cercispinosa* (GMYC

222 > 1 species, S1 Table). These species were divided into two clades (S5 Fig), spatially segregated into

223 eastern and western Japan of the Fossa Magna region during the late Neogene period (20 to 22 Ma).

224 Recent diversifications for *Nemoura* and *Amphinemura* species within either eastern or western

225 Japanese branches were additionally revealed by TMRCA analysis of *cox1* (see Methods). *A. decemseta*

226 ranging from 3 to 3.5 Ma (95% HPD, 2.8 to 4.1 Ma); *A. zonata*, ranging from 3 to 4 Ma (95% HPD, 3.5

227 to 5 Ma); *A. longispina*, ranging from 3.6 to 4.5 Ma (95% HPD, 3.9 to 5 Ma); *A. megaloba*, ranging from

228 3.5 to 4 Ma (95% HPD, 2.8 to 4 Ma); *N. uenoi*, ranging from 3 to 4 Ma (95% HPD, 3.5 to 4.2 Ma) and *N.*

229 *cf. cercispinosa*, ranging from 3.5 to 4.1 Ma (95% HPD, 3 to 5 Ma), for 1.5% Ma and 3.54% Ma analysis

230 respectively.

231

Phylogeographic pattern between Nemoura from Japan and North America

233   DNA sequences in the Japanese clade of *Indonemoura* (single species, *I. nohirae)* showed a

234 high homology with those in the Alaskan species of *Z. columbiana* (*COI*: KM874174; >93% sequence

235 similarity) and *Z. cinctipes* (H3: EF622600; >98% sequence similarity) based on blastn results. The ML

236 phylogenetic trees for both *cox1* and *H3* (Fig 3) showed that the *Indonemoura* Japanese clade

237 clustered with three North American species (*Z. columbiana*, *Z. cinctipes* and *P. delicatula*) and the

238 *Indonemoura* Mainland China clade clustered with the East Asian Nemouridae genera (*Nemoura*,

239 *Protonemura* and *Amphinemura*). The pairwise nucleotide substitution rate based on *cox1* between

240 the *Indonemoura* Japanese clade and *Zapada* spp. or *P. delicatula* from North America ranged from

241 0.13 to 0.15, whereas a higher pairwise nucleotide substitution rate based on *cox1* of 0.26 was

242 observed between the *Indonemoura* Japanese and Mainland China clades (Table 2).

243

244 **Discussion**

245 We studied mitochondrial *cox1* and nuclear *H3* gene sequences to determine the patterns of

246 diversification and phylogenetic relationships of species belonging to four genera of stoneflies of the

247 Nemouridae family in the Japanese Archipelago. We estimated the divergence among *Nemoura*,

248 *Amphinemura*, *Indonemoura* and *Protonemura* to have occurred in the early and mid-Cretaceous

249 (around 100 Ma), which is compatible with previous studies based on fossil records [56], [57]. Our

250 results suggested that these four genera might have dispersed and colonised different areas of the

251 Eurasian continent—including the Japanese landmasses—when they were still connected to the

252 Eurasian continent. Among the four genera, the diversification of *Indonemoura* occurred earlier (120

253 Ma) than that of the other genera (<100 Ma), suggesting that it is an ancient genus. The geological

254 isolation of colonised areas [58], the long evolutionary time [59] and poor dispersal ability of

255 *Indonemoura* [23], [60] might have accounted for their ancient diversification.

256 Based on the phylogenetic relationships of both molecular markers (*cox1* and *H3*), we

257 observed that the three genera *Nemoura*, *Amphinemura* and *Protonemura* were monophyletic and

258 clustered as three independent groups, as previously observed by morphological systematics [16].

259 However, *Indonemoura* was paraphyletic. This genus was divided into two clades corresponding to

260 the Mainland China and the Japanese clades. Surprisingly, the Japanese clade of *Indonemoura* (single

261 species, *I. nohirae*) clustered together with North American species (*Z. columbiana*, *Z. cinctipes* and *P.*

262 *delicatula*), with a low pairwise nucleotide substitution rate (<0.15). The distribution range of these

263 two North America genera covers North America and Eastern Asia. Previous studies suggested that

264 their distribution could be related to the land connection (i.e. the islands) between Alaska and Eastern

265 Asia [15]. Dispersal by island connectivity between Alaska, the Aleutian Islands, the Kamchatka

266 peninsula and the Kuril islands has been observed in other stonefly families (for instance, *Arcynopteryx*

267 *dichroa*, *Capnia nearctica*, *Mesocapnia variabilis* and *Nemoura arctica*) [61]. However, the distribution

268 of *Indonemoura* on these islands is unknown.

269   The complex history of the geological formation of the Japanese Archipelago may provide a
270   possible alternative explanation. The ancestral Japanese landmasses were located on the borders of
271   four major tectonic plates, of which two are continental plates—the Eurasian plate and the North
272   American plate [4] (S6 Fig). The eastern Japanese landmass was located on the North American plate,
273   whereas the western Japanese landmass was located on the Eurasian plate [5]. The dispersal and
274   colonisation of *Indonemoura* might have occurred from the North American plate to the Eurasian
275   continent or vice versa (from the Eurasian continent to the North American plate) before their
276   geographic separation in an ancient time (around 70 to 80 Ma) [62]. Dispersal events between
277   Eurasian and Japanese landmasses are commonly reported for aquatic insects [10], [63]. Particularly,
278   a dispersal event between North America and the Japanese Archipelago was detected by the
279   phylogenetic relationship of the monophyletic group of caddisflies, *Palaeagapetus* spp. [9]. However,
280   no prior studies have observed speciation events of aquatic insects associated with geological events
281   that occurred in ancient times (>12 Ma). Our result suggests an ancient divergence time and a
282   distribution pattern of *Indonemoura*, consistent with a hypothesis of an ancient colonisation
283   influenced by the connection of the Japanese landmass with the North American plate in the Eurasian
284   continent.

285   Nemouridae species diversification, as has been observed in other species of aquatic insects,
286   such as beetles [10], caddisflies [9], water bugs [14] and mayflies [8], [13], was also observed to be
287   affected by the geological formation of the Japanese Archipelago. The diversification of the
288   Nemouridae species occurred during the Paleogene period (<50 Ma). This geological period is
289   consistent with the movement of landmasses (S6 Fig) about 70 Ma ago [4] and the active geological
290   formation of the Japanese Archipelago around 20 Ma ago [5], which could be the cause of the
291   Nemouridae diversification, as previously reported for the mayfly *Dipteromimus flavipterus* (35 Ma)
292   [2].

293   *Indonemoura nohirae* is the single species of *Indonemoura* on the Japanese Archipelago [25],
294   [26]. The morphology of their terminalia resembles that of *Protonemura* rather than of *Indonemoura*,
295   but the characteristic gill formula justifies their taxonomical classification in *Indonemoura* [25], [26].
296   To date, there are 24 *Indonemoura* species from China [16], [24] and 30 species belonging to the
297   Himalayan and Oriental regions in East Asia [15], [20]. These species are morphologically different
298   from *I. nohirae* in Japan [15], [16], [20], [24], [25], [26]. We hypothesise that the *Indonemoura* species
299   of East Asia could be forming separate phylogenetic clades clustered by geographical regions. For the
300   hypothesis testing, further collection of molecular data on *Indonemoura* from wider areas such as
301   Northeast China, Southeast China, Mongolia, Russia and other countries in Asia is needed in future
302   studies.

303   Eight species (*I. nohirae*, *A. decemseta*, *A. zonata*, *A. longispina*, *A. megaloba*, *N. chinonis*, *N.*

304   *uenoi* and *N. cercispinosa*) showed interesting patterns of intra-species separation into two genetic

305   groups corresponding to eastern and western areas of the Fossa Magna region of Honshu Island (S5

306   Fig). Honshu is the centre of insect biodiversity [10]; apart from its extensive territorial space, it is the

307   main island with a geological history [3], [4], [5]. We found supporting evidence on the genetic

308   diversity of these eight species. We found a larger mean nucleotide substitution rate and haplotype

309   number in the Honshu region than in other islands (Table 1). The mean nucleotide substitution rate

310   and haplotype diversity are indications of biodiversity [64], which could lead to evidence of speciation

311   [65]. Out of eight species, the diversification of six species (*A. decemseta*, *A. zonata*, *A. longispina*, *A.*

312   *megaloba*, *N. uenoi* and *N. cercispinosa*) occurred during the late Neogene period (20 to 22 Ma). This

313   event corresponded with the double-door (i.e. the union of eastern and western Japan; S6 Fig)

314   geological model and the formation of the Itoigawa-Shizuoka tectonic line (ISLT) at around 20 Ma [5],

315   [66]. The speciation of aquatic insects was often observed to be influenced by these two geological

316   events [2]. Additionally, species diversification—from eastern or western Japan of the Fossa Magna

317   region—showed recent diversification events (3 to 5 Ma) corresponding with the formation of the

318   small islands in northeastern or southwestern edge areas of Japan (Fig 1). The northeastern islands

319   created land bridges between the Japanese Archipelago and China or Korea, whereas the

320   southwestern islands connected Taiwan or the Philippines with the Japanese Archipelago [5], [66].

321   This connectivity promoted immigration events in Japan that might have contributed to the formation

322   of the current genetic diversity, as previously observed in mayflies [13] and beetles [10].

323   The evolutionary divergence of the Nemouridae family was promoted by the complex

324   geological formation of the Japanese Archipelago. Despite the different evolutionary rate of both

325   molecular markers, Bayesian analysis found congruence between both markers; however, failed to

326   find congruence with their morphological taxonomy. The main morphological character used for

327   identification of adult stoneflies species is its genital morphology. The evolution of genital morphology

328   is, however, governed by within-population sexual selection rather than environmental or geological

329   history of the locations [67]. Conversely, the genetic variation of natural populations has been

330   observed to be directly associated with environmental [68] and geological variations [2]. Therefore,

331   the genetic variation could reflect an independent course in the evolutionary history of *Indonemoura*

332   than do the morphological characters used for their taxonomy. However, we detected that *N. sanbena*

333   shared haplotypes from different lineages, revealing a possible introgression or incomplete sorting of

334   ancestral polymorphisms [10]. This is an often reported phenomenon in stoneflies [40], [69], [70],

335   which remains as unresolved species. Resolving the problems between the process of evolution of

336 morphological characters and the genetic variation within species will improve our future

337 understanding of the origin of the species and the local species distribution.

338   Finally, our inference of divergence time was based on the coalescent simulation approach.

339 Despite the frequent use of this approach, a biased sampling of lineages and extreme state-dependent

340 molecular substitutions rate heterogeneity are known to potentially cause erroneous inference of

341 divergence time [71]. Therefore, combining node calibrations generated by more than one calibration

342 analyses is recommended [71], [72]. A cautious method such as the combined uses of fossil records

343 and biogeographic ages as employed in our analysis may minimise the risk of such erroneous

344 inference.

345

Acknowledgments

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Table 1. Regional distribution of sample size (n), haplotype richness (h) and mean nucleotide substitution rate of Nemouridae species among the three main islands in Japan, based on mitochondrial DNA (*cox1*) sequences. Total species richness was 26, 23 and 6 for Honshu, Shikoku and Kyushu, respectively.

		Honshu			Shikoku			Kyushu		
Genus	Species	n	h	Nucleotide substitution	n	h	Nucleotide substitution	n	h	Nucleotide substitution
Amphinemura	*A. bulla*	5	4	0.006						
	*A. decemseta**	19	13	0.014	9	5	0.02	3	1	0
	A. dentifera	2	1	0	2	2	0.01			
	A. flavostigma	3	3	0.005	3	2	0.01			
	A. longispina	2	1	0						
	A. megaloba	4	2	0.091	3	1	0			
	*A. zonata**	2	2	0.053	1	1		1	1	
	A. sp. n.	1	1		3	3	0.03			
Indonemoura	*I. nohirae**	9	4	0.059	4	2	0.01	2	1	0
Nemoura	*N. akagii*	2	1	0						
	*N. cf. cercispinosa**	2	2	0.011	13	7	0.01	2	1	0
	*N. chinonis**	2	2	0.126	5	3	0.09	1	1	
	N. fulva	3	3	0.043						
	N. cf. hikosan				2	2	0.1			
	N. longicercia	2	1	0	7	5	0			
	N. naraiensis				2	1	0			
	N. ovocercia	1	1							
	N. redimiculum	1	1		3	3	0.01			
	N. sanbena				2	2	0.5			
	N. shikokuensis				4	1	0			
	N. stratum	2	2	0.027						
	N. speciosa	2	2	0.003						
	N. transversospinosa				6	4	0.01			
	N. uenoi	1	1		2	2	0.01			
	N. yakushimana							2	1	0
	N. sp. n. 1				3	1	0			
	N. sp. n. 2	2	2	0.003						
	N. sp. n. 3	1	1							
	N. sp. n. 4	1	1							
Protonemura	*P. kohnoae*	6	3	0.043						
	P. orbiculata	6	6	0.029						
	P. sp. n				2	2	0.01			
	P. sp. n. 1	1	1							
	P. sp. n. 2	2	1	0						
	P. sp. n. 3	1	1							
	P. sp. n. 4							1	1	

(*) Species found on the three Japanese islands.

531 **Table 2**. Pairwise nucleotide substitution rate based on *cox1* between the East Asian Nemouridae and North American (western USA and Alaskan) species.

|									
East Asia	*Amphinemura* spp.	0							
	Nemoura spp.	0.187	0						
	Protonemura spp.	0.197	0.19	0					
	Indonemoura spp. (China)	0.213	0.193	0.197	0				
	Indonemoura spp. (Japan)	0.197	0.183	0.175	0.260	0			
North America	*Zapada columbiana*	0.178	0.165	0.182	0.190	0.145	0		
	Zapada cinctipes	0.170	0.154	0.156	0.179	0.149	0.133	0	
	Podmosta delicatula	0.202	0.196	0.191	0.205	0.135	0.185	0.201	0

532

533 **Figures**

534
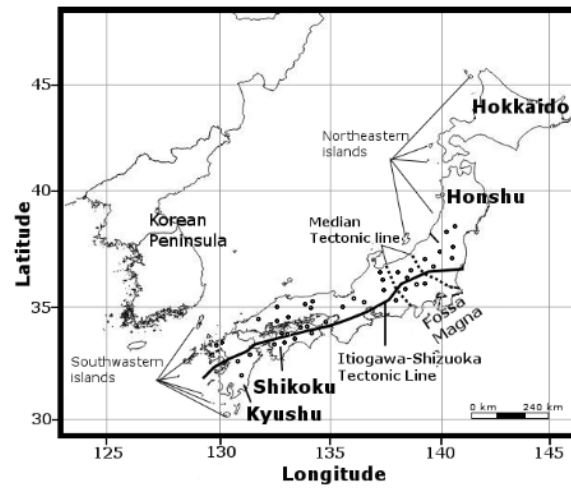
535

536 **Fig 1.** The Japanese islands and distribution of 110 sampling sites from where Nemouridae samples

537 were collected (open circles).
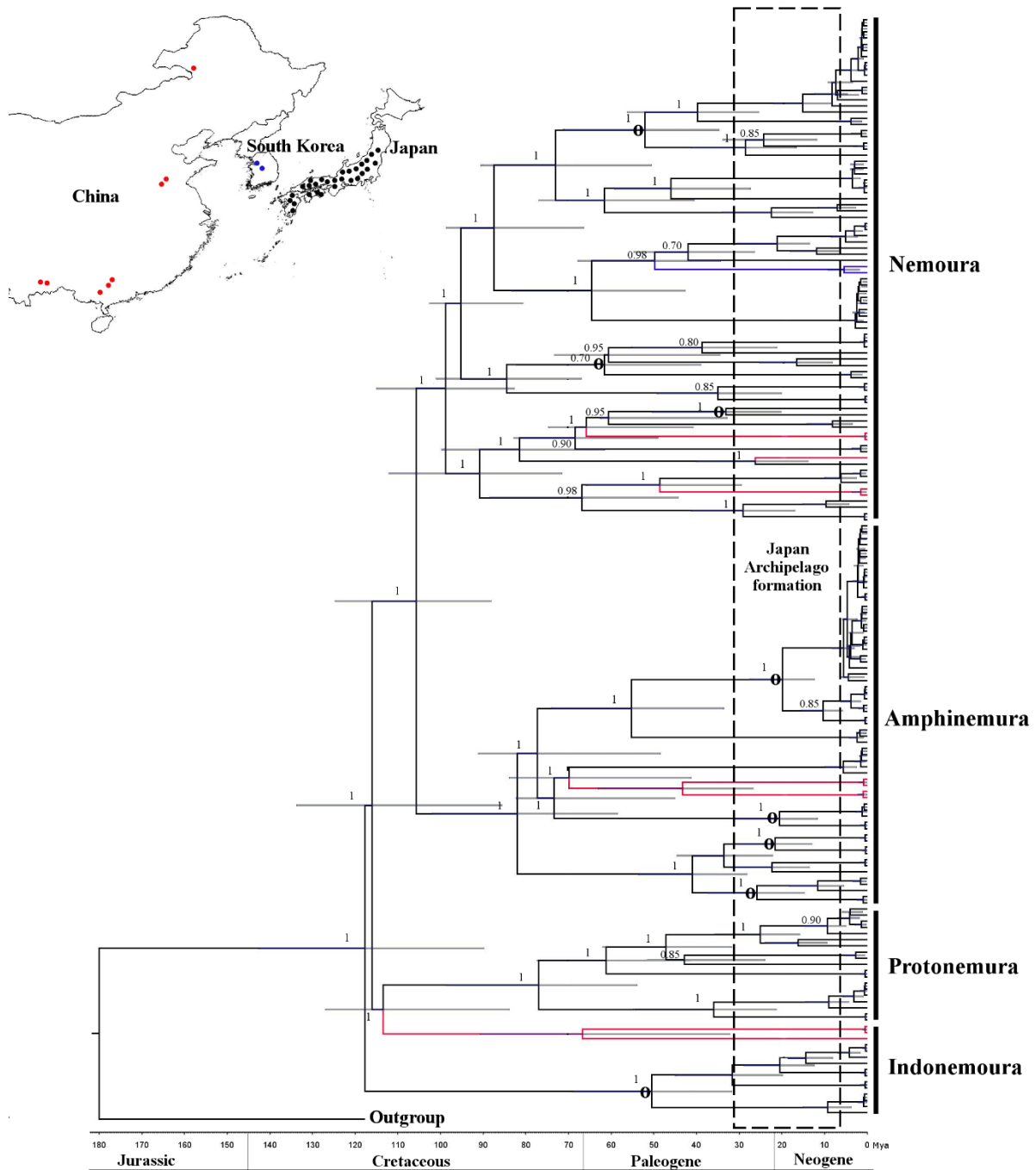
Fig 2. Concatenated Bayesian phylogeny (*cox1* + *H3*) of the East Asian Nemouridae family. The phylogenetic tree nodes were calibrated using 180 Ma based on fossil records + 15 to 30 Ma based on the Japanese Archipelago formation. Calibration and geological time are shown at the bottom of the tree. A 95% HPD is indicated as a horizontal grey bar and posterior probabilities are shown for each node. Circle symbol (**o**) in the nodes indicates intra-species diversification based on the eastern and western Japanese boundaries of the Fossa Magna region. Inserted upper map shows sample site locations for Japan (black), China (red) and South Korea (blue) as dots. Colour branches indicate sample location distribution as shown in the map.

547
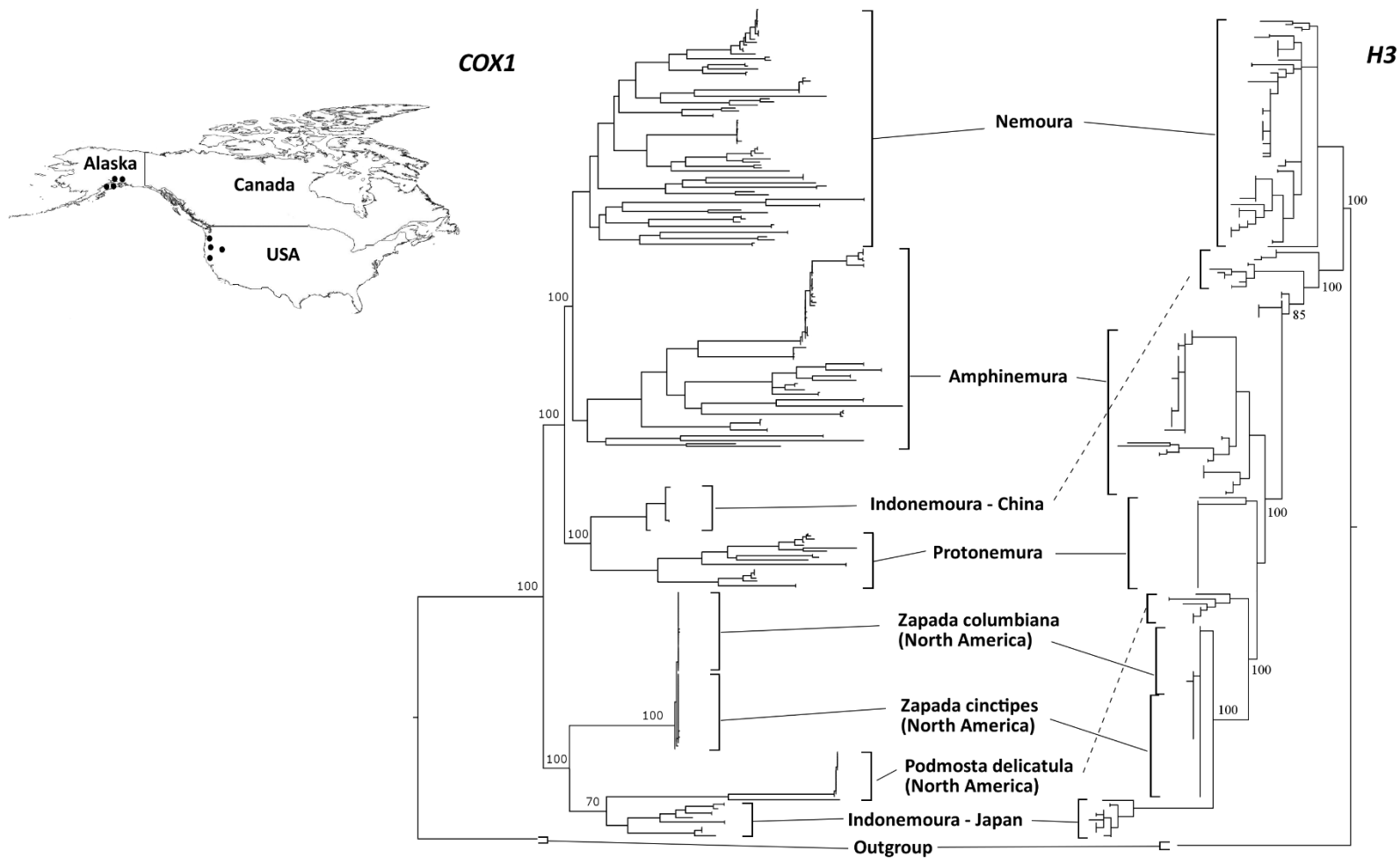
Fig 3. Maximum likelihood trees based on both *cox1* and *H3* markers for comparison between the East Asia Nemouridae family and three North American

Nemourinae species: *Zapada cinctipes*, *Z. columbiana* and *Podmosta delicatula*. Inserted upper map shows sampling site locations in North America (western

USA and Alaska) as black dots.

Supplementary information

S1 Table. Location information of samples of East Asia Nemouridae. Numbers of individuals (N), presences of male (M), female (F) and imago (im), DNA-species delimitation (GMYC).

S1 Fig. Multiple alignment of *cox1* region for the 54 Nemouridae genotypes.

S2 Fig. Multiple alignment of *H3* regions for the 54 Nemouridae genotypes.

S3 Fig. *Cox1* Bayesian trees using three types of node calibration. A – fossil, B – island formation and D – TMRCA.

S4 Fig. *H3* Bayesian tress using two types of node calibration. A – fossil and B – islands formation.

S5 Fig. Concatenated Bayesian phylogeny (*cox1 + H3*) for East Asian Nemouridae family enlarging intra-species diversification in *I. nohirae*, *A. decemseta*, *A. zonata*, *A. longispina*, *A. megaloba*, *N. chinonis*, *N. uenoi* and *N. cf. cercispinosa* (GMYC = 2 species).

S6 Fig. Putative formation of the Japanese Archipelago [2], [3], [4], [5]. (A) Around 30 to 130 Ma, the Japanese landmasses were located in two major tectonic plates from the Eurasian continent. (B) Around 15 to 30 Ma, the Japanese landmasses began to separate from Eurasia and the North American Plates began to separate from the Eurasian continent, and remained separated by a sea zone called Fossa Magna—a geological event called double-door. (C) Current map of the Japanese Archipelago in East Asia, where the names of the main four Japanese islands and the two tectonic lines are shown.