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# Matrilineal Genealogy of *Hynobius* (Caudata: Hynobiidae) and a Temporal Perspective on Varying Levels of Diversity among Lineages of Salamanders on the Japanese Islands

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**Abstract** Previous work found that different Japanese lineages of salamanders had quite different levels of species and genetic diversity. Lineages vary from having one to several species and the extent of genetic variation among lineages differs substantially. Most speciose, genus *Hynobius* contains 18 species and several potential cryptic species. We explore genetic diversity in this genus by combining comprehensive sampling and mitochondrial DNA sequences. Based on this and previous analyses of salamanders, relative times of divergence are employed to evaluate the relationship between age and diversity among the four major lineages whose distributions broadly overlap on the islands. For *Hynobius*, our analyses are congruent with the previously reported high level of cryptic diversity in morphology and allozymes, particularly in species composed of non-sister matrilines. Both species and genetic diversity correlate with the relative ages of the lineages. This correlation indicates that the variation in levels of diversity can be explained, to a considerable extent, by the hypothesis that older insular lineages have accumulated greater diversity. In addition to the Korean Peninsula, *H. leechii* might have survived in another Pleistocene glacial refugium north of the peninsula and this refugium provided a source of colonization after the last glacial maximum.

**Keywords** tempo of diversification, salamander, Japanese Archipelago, *Hynobius*, cryptic species, northern glacial refugium

## 1. Introduction

An understanding of patterns of biodiversity requires the integration of historical and environmental factors (Svenning and Skov, 2005; Hawkins *et al.*, 2006; Donoghue, 2008; Jetz and Fine, 2012). Species diversity in a region is often considered as the product of net diversification rate (speciation rate minus extinction rate), time, and dispersal (Ricklefs, 2004; Wiens and Donoghue, 2004; Rabosky, 2009). Thus, time is an important historical factor when interpreting patterns of species richness. The idea that species richness might be correlated with how long the constituent lineages have been evolving in the area has a long history in the

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evolutionary time hypothesis (Willis, 1922; Fischer, 1960; Stebbins, 1974) and the time-for-speciation effect (Stephens and Wiens, 2003). These perspectives and their variants help explain patterns of species diversity on a variety of spatial scales and in various taxonomic groups (Borges and Brown, 1999; Hawkins *et al.*, 2005, 2007; Pyron and Burbrink, 2009; Roncal *et al.*, 2011), including amphibians (Wiens *et al.*, 2006, 2009; Kozak and Wiens, 2010).

Lineages of salamanders on Japanese islands exhibit quite different levels of species diversity. Five genera occur on islands north of a zoogeographical boundary known as Watase's line between the Palearctic and Oriental regions (Okada, 1927; Tanaka *et al.*, 1975): the hynobiids *Salamandrella keyserlingii*, *Onychodactylus*, and *Hynobius*; the salamandrid *Cynops pyrrhogaster*; and the cryptobranchid *Andrias japonicus*. Among them, the distribution of *S. keyserlingii* is restricted to the northeastern margin of the islands, which is also the southeastern edge of the current distribution of this widespread Eurasian species (Poyarkov and Kuzmin, 2008). The other four genera generally range widely and overlap extensively on the islands (Figure 1). *Andrias japonicus* is endemic to Japan, and it has limited genetic variation throughout its range (Matsui *et al.*, 2008). *Onychodactylus* occurs on Japanese islands and the adjacent Asian mainland. Of the two species endemic to Japan, *O. japonicus* has a high level of genetic variation and possible cryptic taxonomic diversity (Yoshikawa *et al.*, 2008, 2010a, b, 2012; Poyarkov *et al.*, 2012). *Cynops pyrrhogaster* is endemic to Japan and also has a high level of genetic diversity, suggesting the existence of cryptic species (Tominaga *et al.*, 2010; Tominaga *et al.*, in press). *Hynobius* occurs on Japanese islands, Taiwan Island (China), and mainland Asia. Eighteen species of *Hynobius* are endemic to Japan (Frost, 2011) and potential cryptic taxonomic diversity occurs within several species (e.g., Tominaga *et al.*, 2005a; Matsui *et al.*, 2006; Nishikawa *et al.*, 2007). The occurrence of each of these four genera in Japan likely owes to a single colonization event that largely either concurred with or after the Miocene separation of the islands from mainland Asia (Maruyama *et al.*, 1997; Matsui *et al.*, 2008; Yoshikawa *et al.*, 2008; Zhang P. *et al.*, 2008; Tominaga *et al.*, 2010; Li *et al.*, 2011; Zheng *et al.*, 2011).

The various levels of diversity among these lineages may be a function of time since colonization. Although lineage-ages have been estimated previously, cross-study comparisons are complicated by the employment of differing calibration strategies and molecular markers, among-lineage rate variation, and innate uncertainty of the molecular clock itself (e.g., Matsui *et al.*, 2008; Zhang P. *et al.*, 2008; Tominaga *et al.*, 2010). The simultaneous estimation of ages based on a molecular phylogeny containing all these lineages can circumvent this problem when using relaxed molecular clock methods.

Estimations of taxonomic diversity of constituent lineages can facilitate evaluations of the relationship between age and diversity. Phylogenetic analyses based on extensive sampling are available for Japanese lineages of *Andrias*, *Onychodactylus*, and *Cynops* (e.g., Matsui *et al.*, 2008; Yoshikawa *et al.*, 2008; Tominaga *et al.*, in press); *Hynobius* requires evaluation. In Japan, some species of *Hynobius* may contain cryptic taxonomic diversity, especially *H. boulengeri*, *H. naevius*, *H. nebulosus*, and *H. yatsui*, as evidenced by allozymes, morphology, and matrilineal genealogies (e.g., Tominaga *et al.*, 2005a, b, 2006; Matsui *et al.*, 2006; Nishikawa *et al.*, 2007). Previous studies involve a relatively small taxonomic scale, i.e., a few species. On the islands, great morphological similarity among species of *Hynobius* confounds their identification; often locality data are necessary (Nishikawa *et al.*, 2007). Moreover, newly revealed cryptic species are not always the sister lineages of the named species (Donald *et al.*, 2005; Bickford *et al.*, 2007; Seifert, 2009). A genealogical study involving widespread sampling, including not only the Japanese species but also species from other areas, may reinforce the results of previous studies on *Hynobius* and provide new insights. It will not only facilitate the evaluation of diversity of this genus, but also provide an understanding of the dispersal history of its members.

The Korean Peninsula and adjacent areas host six species and proposed species of *Hynobius* (Baek *et al.*, 2011). Except for *H. leechii*, all other species occur on the southern part of the peninsula only. Within its southern range, *H. leechii* has high levels of mitochondrial DNA (mtDNA) diversity (Baek *et al.*, 2011) yet very low levels of diversity in allozymes and mtDNA exist in the northern range of this species (Zeng and Fu, 2004). This "southern richness and northern purity" pattern is consistent with the southern Korean Peninsula providing a glacial refugium for both animals and plants (Hewitt, 2000; Kong, 2000; Serizawa *et al.*, 2002; Zhang H. *et al.*, 2008). Notwithstanding, Zeng and Fu (2004) reported a substantial genetic difference between the southern (two individuals) and northern forms of *H. leechii*, thus indicating a possible northern refugium outside the

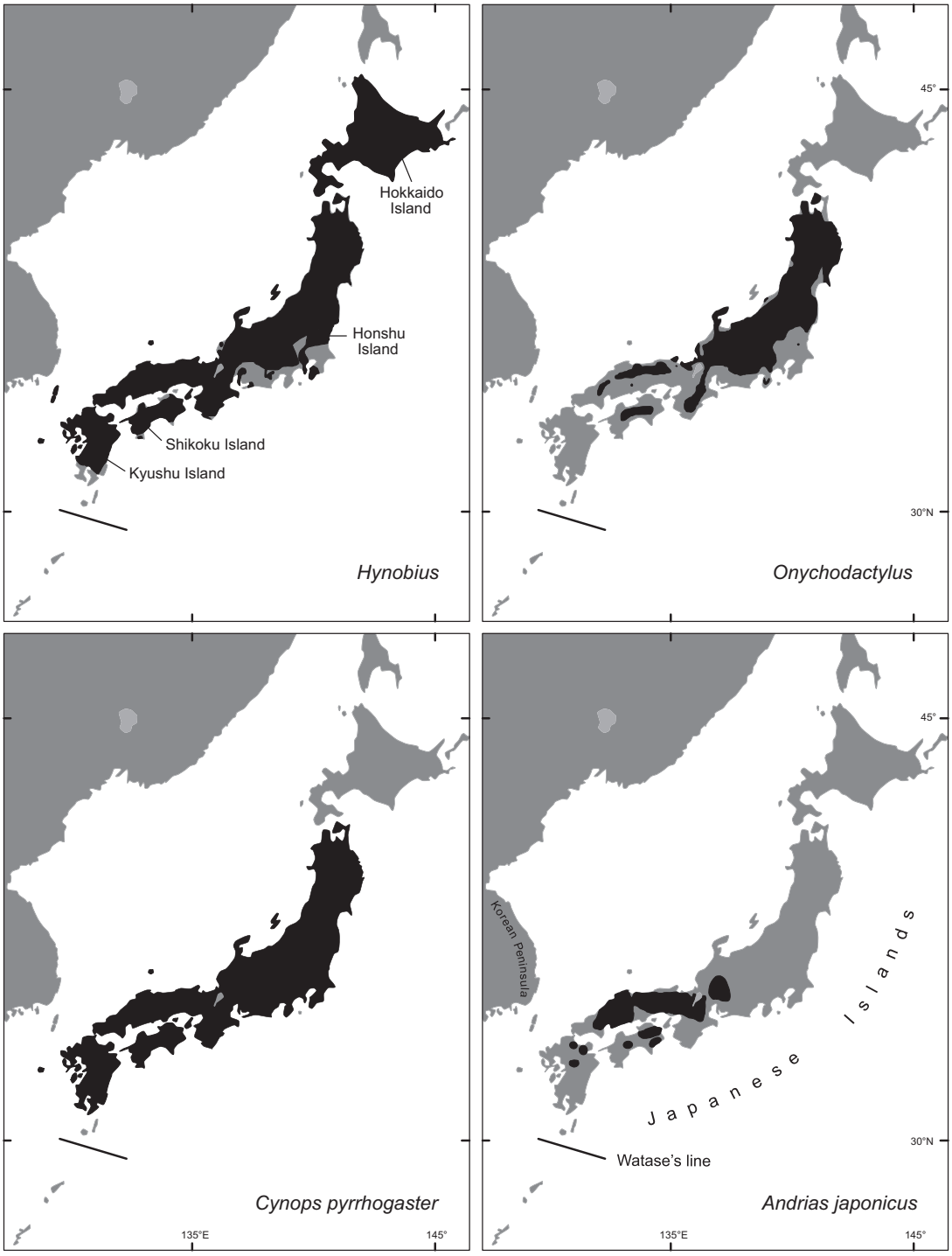



**Figure 1** Distribution of salamanders on the Japanese islands. Distributions (black) of the species of *Hynobius* and *Cynops pyrrhogaster* were obtained from IUCN (2011). The ranges (black) of *Onychodactylus* and *Andrias japonicus* were mapped following Yoshikawa *et al.* (2008) and Matsui *et al.* (2008), respectively.

peninsula (Provan and Bennett, 2008), as proposed for rodents (Serizawa *et al.*, 2002; Lee *et al.*, 2008).

Herein, we first explore the matrilineal history and diversity of the genus *Hynobius* based on comprehensive sampling and apply the results to propose taxonomic hypotheses. On the basis of this and previous molecular analyses of salamanders, we simultaneously estimate the relative divergence times to evaluate the relationship between lineage-age and current diversity among salamanders on the Japanese islands. The species *S. keyserlingii* is not included in this study because its restricted insular distribution is considerably isolated from

the ranges of most other lineages. Finally, we examine the possibility of a northern glacial refugium for *H. leechii* using increased sample sizes and sampling sites from both the northern and southern parts of its distribution.

## 2. Material and methods

**2.1 Taxon sampling and DNA sequencing** Genealogical analyses of *Hynobius* used a total of 368 individuals from 30 of the 32 species (Frost, 2011) as the ingroup. Three sets of DNA sequence data were available for this genus. The first included the entire mitochondrial genome (e.g., Zhang *et al.*, 2006; Zheng *et al.*, 2011). Second, data from the mitochondrial gene *cyt b* included 1141 bp, the full length (e.g., Matsui *et al.*, 2007; Lai and Lue, 2008; Sakamoto *et al.*, 2009; Baek *et al.*, 2011). The third data set included two mitochondrial fragments: a *12S–16S*, ~1075 bp fragment including the intervening tRNA gene (GenBank accession No. AY915973–96) and a ~1455 bp fragment consisting of the complete *ND2* and part of *COI* genes and the tRNA genes between them (AY915925–48) (Macey *et al.*, 2005, unpublished data). All these GenBank sequences of *Hynobius* as of February, 2012 were included in the analyses. Several individuals with *cyt b* sequence data also had a *12S* sequence (Tominaga *et al.*, 2006) that overlapped substantially with the *12S–16S* fragment; these *12S* sequences were included in the analysis. We sequenced the mitochondrial genomes of one individual each of five species, *cyt b* from 50 individuals representing 23 sampling sites plus three individuals without precise locality data, and the *12S–16S* fragment of one individual (JQ929919–77). Mitochondrial genomes of five species (*H. hidamontanus*, *H. kimurae*, *H. lichenatus*, *H. nigrescens*, and *H. tsuensis*) were sequenced for the first time with the purpose of including more distinct full-length sequences in the supermatrix approach. The other samples sequenced concentrated on mainland species, especially *H. leechii*. Two species of *Batrachuperus* and *Liua* were included in the outgroup based on the current phylogeny (Peng *et al.*, 2010; Zheng *et al.*, 2011). Details of the sampling and PCR primers were presented in Supplementary Material 1. No DNA sequences were available from *H. hirosei* and *H. turkestanicus*.

Sequences of all nine mitochondrial light-strand encoded genes were converted into their complementary strand. Alignment was conducted with ClustalX v. 1.83 (Thompson *et al.*, 1997) and checked by eye. Homologies of non-coding genes were checked against the secondary structures of tRNAs determined using tRNAscan-SE v. 1.21 (Lowe and Eddy, 1997) and the rRNA secondary structures of *Xenopus laevis* (Cannone *et al.*, 2002) and the salamander *Ambystoma mexicanum* (Wuyts *et al.*, 2004). Amino acid sequences were used to confirm homologies for coding regions. Sites of questionable homology were excluded from analysis.

**2.2 Molecular phylogenetic analysis of the genus *Hynobius*** All 13 protein-coding, two rRNA, and 22 tRNA mitochondrial genes were analyzed in combination. Because no overlaps occurred between sequences of some haplotypes, two datasets were analyzed separately, each composed of different subsets of taxa based on aligned sequences (Supplementary Material 2). In the larger dataset, Data-L, all haplotypes had an overlapping fragment of *cyt b*. In the smaller dataset, Data-S, *12S* overlapped. A six-partition strategy was applied to both datasets. A separate partition was defined for each codon position from all protein-coding genes, another for each rRNA gene, and one partition for the concatenated tRNA genes (Mueller *et al.*, 2004; Zhang and Wake, 2009). The Bayesian information criterion (BIC) and the corrected Akaike information criterion (AICc) implemented in jModelTest v. 0.1.1 (Posada, 2008) were used to select an evolutionary model that best fit each dataset partition (Posada and Buckley, 2004; Tamura *et al.*, 2011).

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted on both datasets. BI was performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) on the CIPRES web portal (Miller *et al.*, 2010). Four Monte Carlo Markov chains (MCMC) were used to obtain 20 million generations. Six independent runs were performed to ensure that analyses were not trapped in local optima. Trees were sampled every 1000 generations and the last 10 000 sample trees were used to construct a majority rule consensus tree and the frequency of nodal resolution was termed a Bayesian posterior probability (BPP). ML analyses were conducted using RAxML v. 7.2.6 (Stamatakis, 2006). The rapid hill-climbing algorithm (Stamatakis *et al.*, 2007) was used and 200 inferences were executed. Nodal support was estimated with nonparametric bootstrap proportions (Felsenstein, 1985) involving 1000 replicates. In addition, the Shimodaira-Hasegawa (S-H) test (Shimodaira and Hasegawa, 1999) was conducted using RAxML to evaluate the significance of alternative topologies.

For *H. leechii*, an unrooted network approach was employed to visualize associations among the haplotypes of *cyt b*. At the 95% confidence level, the statistical parsimony analysis was performed using TCS v. 1.21 (Clement *et al.*, 2000).

**2.3 Estimating sequence divergence** In *Hynobius*, genetic diversity assessments for *cyt b* and *12S* used uncorrected nucleotide p-distances calculated in MEGA v. 5.05 (Tamura *et al.*, 2011) based on the alignment for genealogical analysis. Pairwise distances were calculated for all haplotypes and between-group mean distances were obtained when necessary. Substantial p-distances between *H. leechii* and *H. yangi*, *H. boulengeri* and *H. kimurae*, and *H. amjiensis* and *H. yiwuensis* served as conservative interspecific reference points.

**2.4 Molecular clock analysis** A total of 38 species of salamanders were selected as representatives for the clock analysis. Most hypothesized divergence events, i.e., nodes in phylogenetic trees, leading to Japanese lineages of the genera *Andrias*, *Cynops*, *Hynobius*, and *Onychodactylus* were included; several weakly supported events were not included to avoid historical uncertainty. In addition to the *12S–16S* and *cyt b* fragments, only the mtDNA fragment *ND1–COI* (approximately 2040 bp before alignment) was also available for all these species as of February, 2012. These three fragments were concatenated for analysis. Two anuran species, *Xenopus tropicalis* and *Ascaphus truei*, were selected as outgroup members (Cannatella *et al.*, 2009; Zhang and Wake, 2009). Details of sampling for the molecular clock analysis were provided in Supplementary Material 3. We employed the same methods of sequence alignment, dataset partitioning, and selection of a substitution model as noted above. The sequence alignment was presented in Supplementary Material 2.

We used a reference topology involving 38 sampled species of salamanders for the molecular clock analysis. This consisted of results that were congruent for previous molecular studies and our analyses. Compared with our three-fragment dataset, Data-MC, some other datasets had more comprehensive taxon sampling for particular lineages and/or more loci (i.e., the complete mitochondrial genome) (Vieites *et al.*, 2007; Matsui *et al.*, 2008; Yoshikawa *et al.*, 2008; Zhang P. *et al.*, 2008; Zhang and Wake, 2009; Zheng *et al.*, 2011). The likelihood ratio test rejected ($P < 0.001$) the hypothesis that our data evolved according to a strict molecular clock. Therefore, two widely used, relaxed molecular clock methods were adopted: the penalized likelihood approach (PL; Sanderson, 2002) and the Bayesian approach developed by Thorne, Kishino, and their colleagues (TK; Thorne *et al.*, 1998; Kishino *et al.*, 2001; Thorne and Kishino, 2002).

No calibrations were used in the molecular clock analysis because we focused on the temporal sequence of divergence. Given the poor fossil record for salamanders, calibration would have used a large probability distribution (Anderson, 2008, 2012; Zhang and Wake, 2009) and estimated divergence times of this group would have varied broadly (e.g., Vieites *et al.*, 2009). Such uncertainty would likely obscure the temporal sequence of interest. As a result, and also because the uncertainty in the root age would be a major source of uncertainty in the estimates of node ages (Wiens, 2007), the ingroup root, the Cryptobranchoidea–Salamandroidea split, was fixed with an arbitrary number.

For PL, branch lengths in the ML framework (Schwartz and Mueller, 2010) were estimated on the reference topology using RAxML. The PL analysis was performed using r8s v. 1.71 (Sanderson, 2003), in which estimates had two decimal places. The ingroup root was fixed at the arbitrary value of 500 to facilitate the comparison of estimates. The smoothing parameter value was set to 16, which was selected from values between 0.01 and 10 000 by conducting a cross-validation test. To assess error levels in estimates, 200 partitioned bootstrap replicate datasets were generated (in RAxML) and analyzed with the help of Torsten Eriksson's r8s-bootkit available at http://www.bergianska.se/index_forskning_soft.html (Sanderson and Doyle, 2001). In doing this, the cross-validation test was performed for each bootstrapped dataset.

The TK analysis followed the manual of Rutschmann (2005) and was implemented with PAML v. 4 (Yang, 2007) and Multidivtime (Thorne and Kishino, 2002). The ingroup root was constrained to a value between 4.999 and 5.001 because calibrations could not be fixed in Multidivtime and an ingroup root age prior of between 0.1 and 10 time units was recommended in the Multidivtime readme file. The priors for rate of evolution at the root branch and the standard deviation (SD) of the rate were set to the same value, which was 1/2 of the mean of ML distances between species-pairs descended from the root node divided by 5. The priors for the Brownian motion constant and the SD of the constant were both set to 1. The beta prior on proportional branch depth was set to 1. The number of samples, cycles between samples, and cycles before the first sample were set to values of 10 000, 100, and 2 000 000, respectively. The analysis was run twice and the results were compared to ensure that the MCMC reached stationarity.

As a comparison of proportional time estimates for different divergence events, ratios between the estimates were calculated. Calculation of the ratio used estimates from PL based on Data-MC and mean estimates from the TK approach. The 95% confidence interval of the ratio

was estimated using either 200 bootstrap replicates (PL) or the 10 000 samples of the MCMC (TK). A ratio value was first calculated for each bootstrap replicate or sample, and then the confidence interval was calculated as either bootstrap mean ± 1.96 SD (PL; normality not rejected at a 0.05 level by the Kolmogorov-Smirnov test implemented in SPSS v. 12.0) or by sorting the 10 000 resulting values and reporting the 250$^{th}$ and 9750$^{th}$ values (TK).

## 3. Results

**3.1 Molecular phylogenetic analysis** Data-L contained 263 haplotypes and 15 069 nucleotide sites, of which 5061 sites were variable, and 3780 were potentially parsimony-informative among the ingroup members. Among the haplotypes, 23 were based on complete or nearly complete mitochondrial genomes and 262 overlapped *cyt b* at 637 sites. The exception, Hkim-1, contained 370 sites of the overlapping region. Data-S had 53 haplotypes and 15 069 positions, of which 5081 were variable and 3870 were potentially parsimony-informative among the ingroup members. A total of 23 haplotypes were based on mitochondrial genomes, 46 overlapped by 2334 positions, and all haplotypes overlapped *12S* at 532 sites. In both datasets the overlapping regions between coding genes *ATP8* and *ATP6* (ten positions), *ND4L* and *ND4* (seven positions), and *ND5* and *ND6* (15 positions) were treated as a second codon position. RAxML applied one substitution model to all DNA data partitions and the GTR+I+G model was used. Models with a proportion of invariable sites were mostly selected for individual partitions. For BI, a same set of model parameters were selected by BIC and AICc for Data-L, but different parameters were selected by the two criteria for Data-S. Consequently, separate BI analyses of Data-S used parameters selected by BIC and AICc and the results were compared. Substitution models selected for individual dataset partitions were listed in Supplementary Material 4.

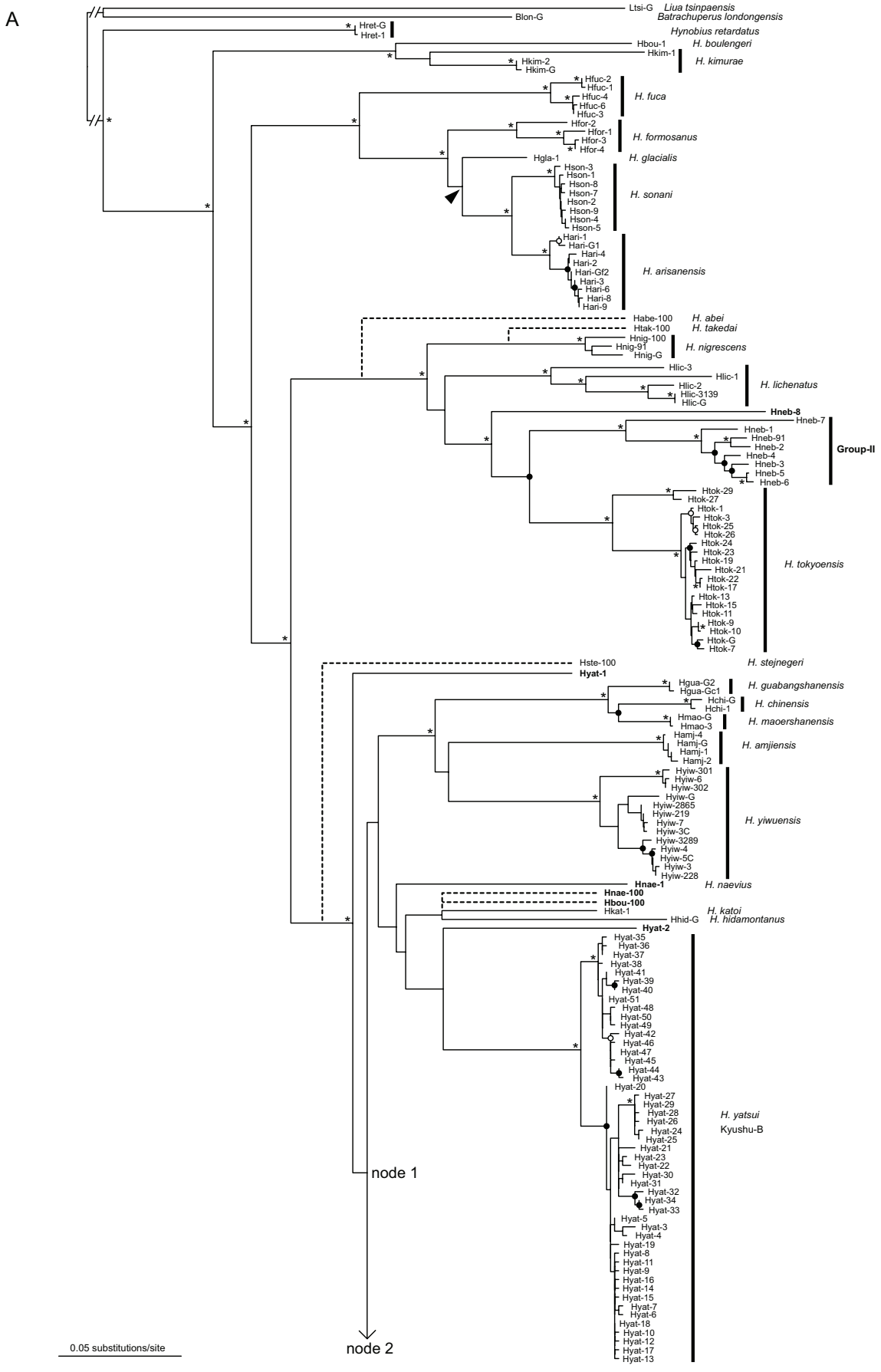
The ML and BI analyses of Data-L produced two very similar topologies with most major lineages being well-supported. As the only notable difference, *H. glacialis* formed the sister group of *H. arisanensis* + *H. sonani* on the ML tree (Figure 2), while the relationship among *H. glacialis*, *H. formosanus*, and *H. arisanensis* + *H. sonani* remained unresolved on the BI tree. All the other differences involved poorly supported, intraspecific relationships, mostly within particular lineages. Both approaches resolved two lineages for *H. leechii*, termed A and B. Lineage A was distributed in the southern part of the species' range. In Lineage B, all the three haplotypes from the northern area formed a sublineage (Figure 2, Northern Sublineage) that clustered within samples from the southern area.

The ML and BI analyses of Data-S produced nearly identical topologies in which most nodes were well-supported. Independent BI analyses using model parameters selected by BIC and AICc produced identical topologies and similar BPPs. Only one difference emerged between the ML and BI trees; the ML tree (Supplementary Material 5) resolved Hyat-1 as the sister group of the lineage containing haplotypes Hhid-G, Hbou-100, and Hnae-100. In the BI tree, the relationships among Hyat-1, Hnae-1, and the lineage containing Hhid-G, Hbou-100, and Hnae-100 were unresolved.

Weakly-supported alternative topologies were obtained for haplotypes Hnae-1, Hneb-G, and Hyat-1 from Data-L and Data-S yet these trees were mostly compatible (Figure 2; Supplementary Material 5). Thus, some samples included only in Data-S were integrated into the Data-L tree (Figure 2). Combined, a total of 39 major lineages of *Hynobius* were identified. Some haplotypes of *H. boulengeri*, *H. naevius*, *H. nebulosus*, and *H. yatsui* did not cluster with conspecific samples. For *H. nebulosus*, the forced unification of all haplotypes from Kyushu Island (Hneb-G, Hneb-9, and Hneb-100) in the Data-S tree was not rejected by the S-H test ($P > 0.05$). The same result occurred for Hneb-G and Hneb-9 in the tree from Data-L ($P > 0.05$). Our gene trees were similar to those of previous studies (Matsui *et al.*, 2007; Lai and Lue, 2008; Nishikawa *et al.*, 2010; Baek *et al.*, 2011; Li *et al.*, 2011; Zheng *et al.*, 2011).

For *H. leechii*, after excluding sites with missing data, the network analysis for *cyt b* contained 740 sites and 27 haplotypes. It resolved two groups corresponding to Lineage A and Lineage B. In the network corresponding to Lineage B (Figure 3), the three northern haplotypes clustered together and connected to the interior southern haplotypes through 10 unobserved intermediate haplotypes.

**3.2 Sequence divergence** Pairwise and between-group p-distances were estimated for overlapping regions of *cyt b* (637 sites) and *12S* (532 sites) (Table 1). The interspecific reference distances ranged of 6.31%–12.66% (*cyt b*) and 1.69%–3.01% (*12S*). Similar levels of divergence were observed when Hnae-100, Hyat-1, Hyat-2, Hbou-100, Hneb-8, and Group-II (Figure 2) were compared with other lineages. These six lineages were from the four species that did not cluster into a single matriline.


A
Ltsi-G
Liua tsinpaensis
Blon-G
Batrachuperus londongensis
Hret-G
Hret-1
Hynobius retardatus
Hbou-1
H. boulengeri
Hkim-1
H. kimurae
Hkim-2
Hkim-G
Hfuc-2
Hfuc-1
Hfuc-4
H. fuca
Hfuc-6
Hfuc-3
Hfor-2
Hfor-1
H. formosanus
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Hfor-4
Hgla-1
H. glacialis
Hson-3
Hson-1
Hson-8
Hson-7
H. sonani
Hson-2
Hson-9
Hson-4
Hson-5
Hari-1
Hari-G1
Hari-4
Hari-2
Hari-Gf2
H. arisanensis
Hari-3
Hari-6
Hari-8
Hari-9
Habe-100
H. abei
Htak-100
H. takedai
Hnig-100
Hnig-91
H. nigrescens
Hnig-G
Hlic-3
Hlic-1
H. lichenatus
Hlic-2
Hlic-3139
Hlic-G
Hneb-8
Hneb-7
Hneb-1
Hneb-91
Hneb-2
Group-II
Hneb-4
Hneb-3
Hneb-5
Hneb-6
Htok-29
Htok-27
Htok-1
Htok-3
Htok-25
Htok-26
Htok-24
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Htok-22
Htok-17
Htok-13
Htok-15
Htok-11
Htok-9
Htok-10
Htok-G
Htok-7
Hste-100
H. stejnegeri
Hyat-1
Hgua-G2
Hgua-Gc1
H. guabangshanensis
Hchi-G
Hchi-1
H. chinensis
Hmao-G
Hmao-3
H. maoershanensis
Hamj-4
Hamj-G
H. amjiensis
Hamj-1
Hamj-2
Hyiw-301
Hyiw-6
Hyiw-302
Hyiw-G
Hyiw-2865
Hyiw-219
H. yiwuensis
Hyiw-7
Hyiw-3C
Hyiw-3289
Hyiw-4
Hyiw-5C
Hyiw-3
Hyiw-228
Hnae-1
H. naevius
Hnae-100
Hbou-100
Hkat-1
H. katoi
Hhid-G
H. hidamontanus
Hyat-2
Hyat-35
Hyat-36
Hyat-37
Hyat-38
Hyat-41
Hyat-39
Hyat-40
Hyat-51
Hyat-48
Hyat-50
Hyat-49
Hyat-42
Hyat-46
Hyat-47
Hyat-45
Hyat-44
Hyat-43
Hyat-20
Hyat-27
Hyat-29
Hyat-28
H. yatsui
Hyat-26
Kyushu-B
Hyat-24
Hyat-25
Hyat-21
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Hyat-22
Hyat-30
Hyat-31
Hyat-32
Hyat-34
Hyat-33
Hyat-5
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node 2
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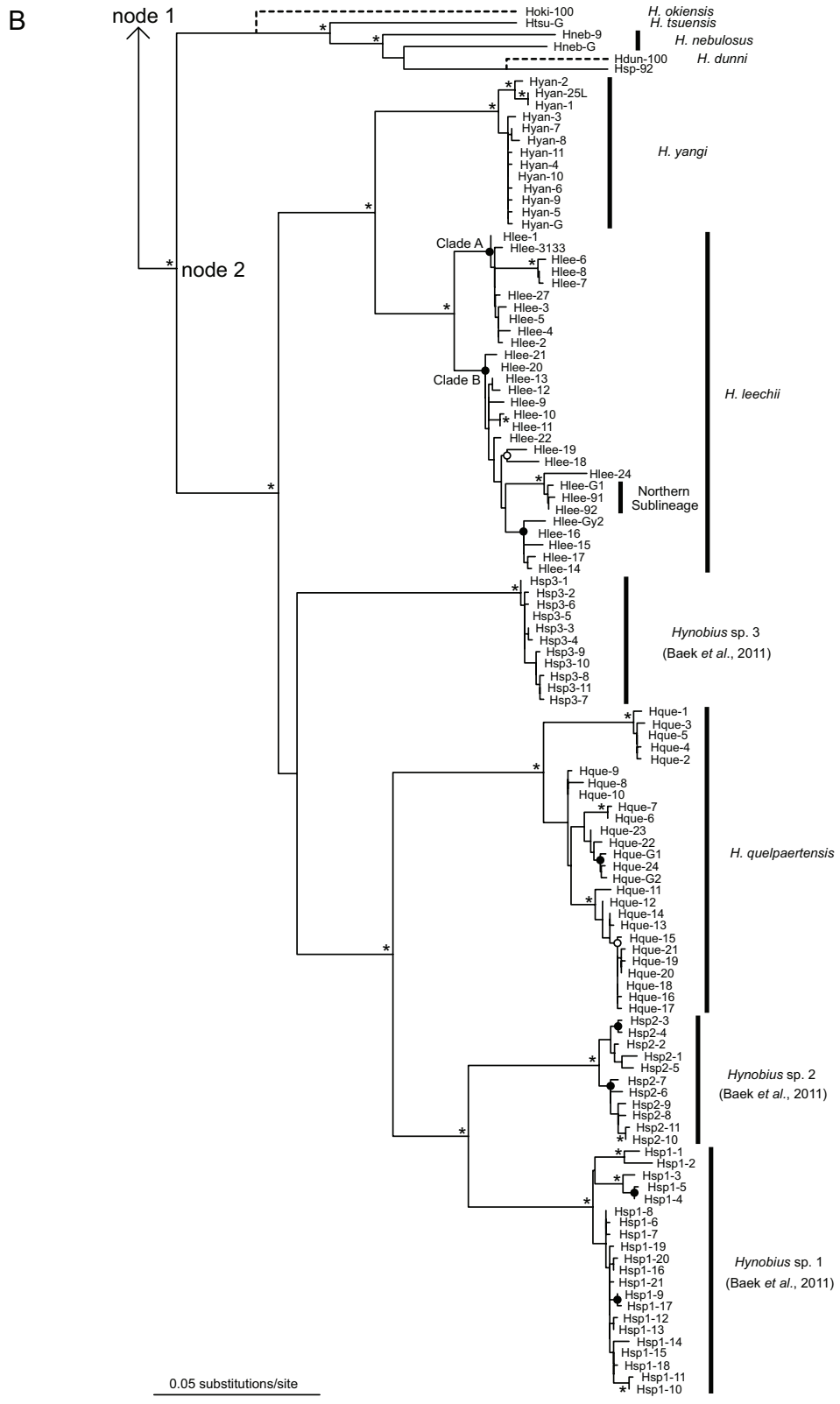


**Figure 2** The matrilineal genealogy of *Hynobius* inferred from a maximum likelihood (ML) analysis of the large mitochondrial dataset Data-L in which all haplotypes overlapped the fragment for *cyt b*. Nodes with ML bootstrap proportions (BP) ≥ 90 and Bayesian posterior probabilities (BPP) ≥ 95 are indicated as asterisks. Nodes with 90 > BP ≥ 70 and BPP ≥ 95 and nodes with 90 > BP ≥ 70 and 95 > BPP ≥ 80 are marked by closed and open circles, respectively. Vertical bars indicate species designation or lineage assignment. Dashed branches indicate mapped samples from small dataset Data-S where the fragments of *12S* overlapped. Arrow indicates a node not recovered in the Bayesian inference analysis. Haplotype names correspond with those in Supplementary Material 1.

**3.3 Proportional time estimates** Proportional time estimates for four splitting events were compared, including the basal split in *Hynobius*, the split between the Japanese lineage of *Onychodactylus* and *O. zhaoermii* from northeastern China, between *C. pyrrhogaster* and *C. ensicauda*, and between *A. japonicus* and *A. davidianus*. For PL, branch lengths were estimated with the GTR+I+G model (Supplementary Material 4). PL and TK approaches yielded similar estimates for these events and the same three-level temporal sequence of the events (Figure 4). The basal split in *Hynobius* predated all others. Splits within *Onychodactylus* and *Cynops* occurred near simultaneously and were relatively recent events, ranging from 0.54 to 0.67 times that of *Hynobius*. The split for *Andrias* ranged from 0.22 to 0.24 of that for *Hynobius*, and thus it was the youngest. Differences between these divergences time were statistically significant; the 95% confidence interval of a ratio between two levels of time was less than one. Several divergences between Japanese species of *Hynobius* were temporally close to the basal split of this genus in the PL and TK analyses (Figure 4).

**Table 1** Estimated nucleotide distances among salamanders of the genus *Hynobius*. Haplotype and lineage names correspond with names in Figure 2 and Supplementary Material 1.

| Comparison | Nucleotide p-distance (%) | |
|---|---|---|
| | *cyt b* (637 sites) | *12S* (532 sites) |
| *H. leechii* – *H. yangi* | 6.31[a] | 1.69[a] |
| *H. boulengeri* – *H. kimurae* | 8.95[a] | 3.01[a] |
| *H. amjiensis* – *H. yiwuensis* | 12.66[a] | 1.72[a] |
| Hnae-1 – other haplotypes | ≥ 10.20 | ≥ 1.88 |
| Hnae-100 – other haplotypes | — | ≥ 2.44 |
| Hyat-1 – other haplotypes | ≥ 9.89 | ≥ 1.88 |
| Hyat-2 – other haplotypes | ≥ 8.32 | ≥ 2.63 |
| Hbou-100 – other haplotypes | — | ≥ 2.26 |
| Hneb-8 – *H. tokyoensis* | 13.44[a] | — |
| Hneb-8 – Group-II | 11.19[a] | — |
| Group-II – *H. tokyoensis* | 11.19[a] | — |
| Hneb-G – Hneb9 | 7.85 | — |
| Hneb-G – (Hneb9 + Hneb100) | — | 1.22[a] |

[a] Between-group mean distances.

## 4. Discussion

**4.1 Age-diversity relationship in salamanders of the Japanese islands** Time is critical for understanding the drivers of diversity (Wiens *et al.*, 2009; Rabosky, 2012; Kozak and Wiens, 2012). Among the four lineages of Japanese salamanders studied, lineage-age estimates are positively related to species richness and phylogenetic diversity.

In East Asia, the ancestral distribution of extant *Hynobius* is estimated to occur on land masses of the current Japanese islands (Li *et al.*, 2011). Thus, it is likely

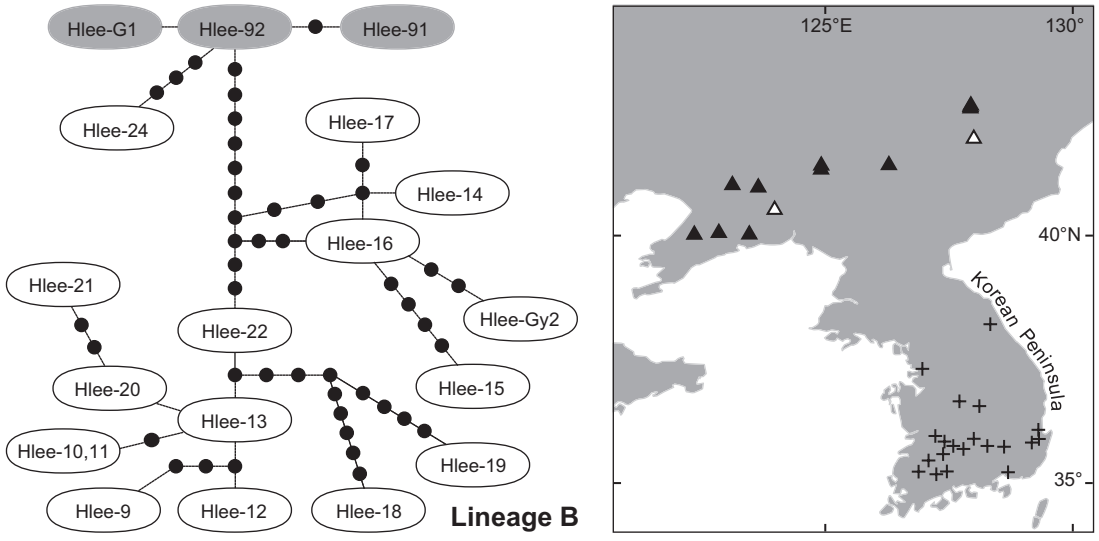


**Figure 3** Network for *cyt b* haplotypes from Lineage B of *Hynobius leechii* and sampling sites of this species. Lines represent single mutational changes. Ovals are sampled haplotypes. Closed circles represent haplotypes that are necessary intermediates but not encountered. Shaded ovals are haplotypes found in the northern sampling sites indicated by triangles. Occurrences of haplotype Hlee-92 are indicated by closed triangles. Haplotype names correspond with those in Figure 2 and Supplementary Material 1. Two haplotypes used in the genealogical analysis are identical after excluding positions with missing data. Locality data for three specimens are not precise enough to be included in the map (Supplementary Material 1).

that this area was colonized by *Hynobius* no later than the basal split (node H, Figure 4) among East Asian members of the genus, which is dated at about 11–23 million years ago (Zheng *et al.*, 2011). As the oldest lineage, this genus gradually diverged long ago to form 18 species plus some possible cryptic species in Japan (Figures 2, 4). In contrast to *Hynobius*, the genera *Andrias*, *Onychodactylus*, and *Cynops* each have one endemic lineage (one or two species) on the islands. All of them nest within relatives occurring outside the islands (Matsui *et al.*, 2008; Yoshikawa *et al.*, 2008; Zhang P. *et al.*, 2008; Tominaga *et al.*, 2010). Consequently, the divergence times between these endemic lineages and their congeneric sister taxa (C and A, Figure 4) or close relatives (O, Figure 4; see below) are used here as indicators of how long each lineage has been evolving on land masses of the islands.

The lineages of *Onychodactylus* and *C. pyrrhogaster* have moderate periods of evolutionary time and corresponding levels of diversity. Date estimates for splitting events O and C are significantly younger in being 0.54 to 0.67 times less than that for H (Figure 4). In *O. japonicus sensu lato*, using complete sequences of *cyt b*, Yoshikawa *et al.* (2008) recognized four considerably differentiated lineages (p-distances 5.5%–9.6%). They suggested the presence of cryptic species. In support of this, extensive genetic and morphological variation occurs in this species and several candidates of cryptic species and one new species, *O. nipponoborealis*, were

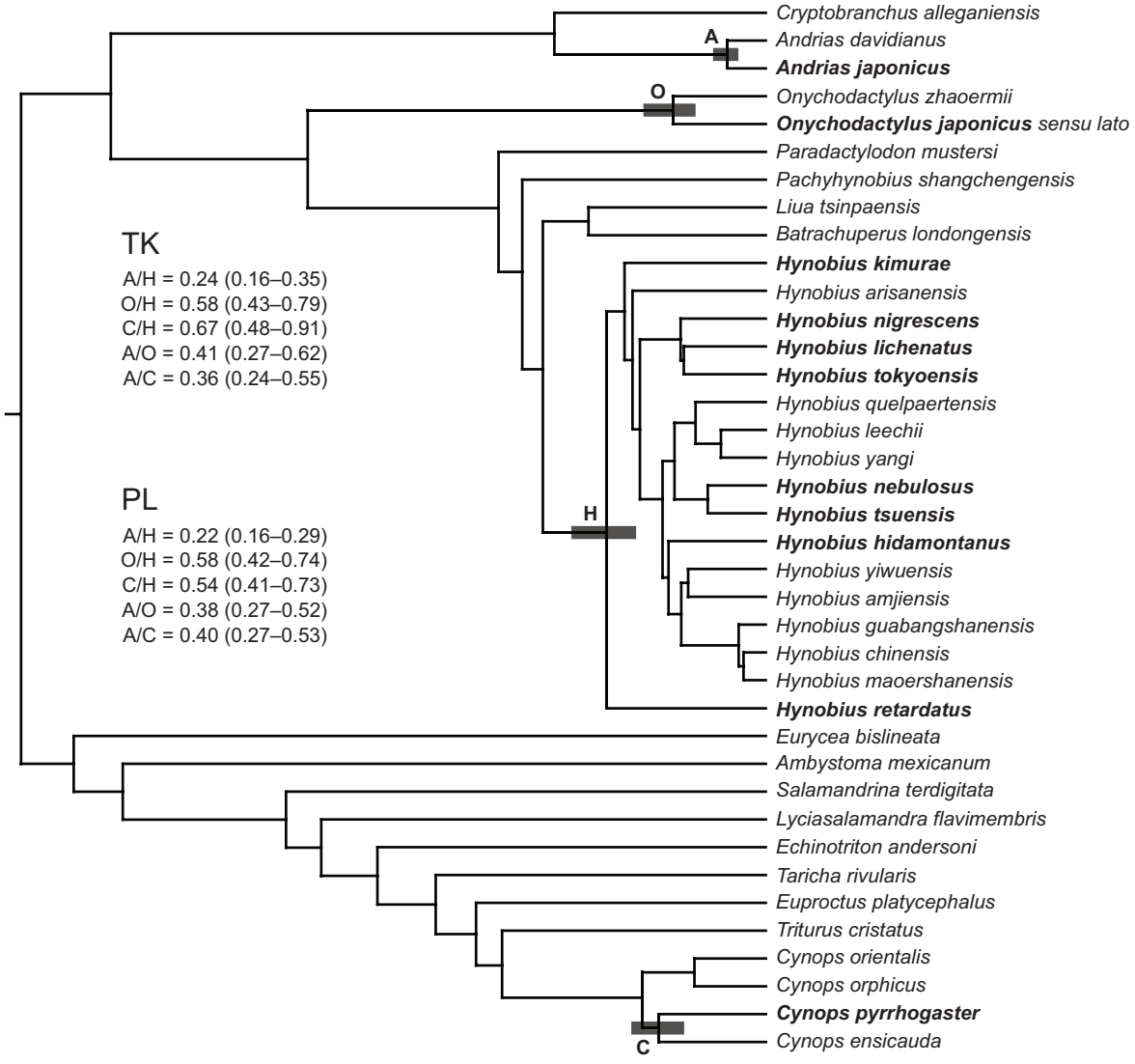


**Figure 4** The time-calibrated tree of salamanders based on the mitochondrial dataset Data-MC containing 3693 nucleotide positions. Proportional times for nodes were estimated using the Bayesian approach developed by Thorne, Kishino, and their colleagues (TK). Gray bars through the nodes indicate 95% highest posterior densities for estimates. Numbers in parentheses are 95% confidence intervals. PL = the penalized likelihood approach. Bold species names indicate species that occur on the Japanese islands north of Watase's line. The outgroup is not shown.

reported in follow-up studies (Yoshikawa *et al.*, 2010a, b, 2012; Poyarkov *et al.*, 2012). The same magnitude of difference for the complete *cyt b* sequences also occurs in *C. pyrrhogaster*, based on limited samples of this wide-ranging species (Tominaga *et al.*, 2010). Based on a comprehensive sampling, Tominaga *et al.* (in press) recently suggested that they were actually four species. *Andrias japonicus* has the shortest period of evolutionary time and lowest level of genetic diversity. Date estimates for the split of *Andrias* (A) range from 0.22 to 0.24 times of the estimate for *Hynobius*, and this is significantly later than the splits of *Onychodactylus* and *Cynops*. Compared with its sister species in mainland Asia, *A. japonicus* exhibits less genetic divergence (Murphy *et al.*, 2000; Matsui *et al.*, 2008). These correlations indicate that the varying levels of diversity among lineages of salamanders on the Japanese islands likely owe, to a considerable extent, to the amount of time that each lineage has been evolving in the region.

Our molecular clock analyses cover a time-span of about 200–300 million years (Roelants *et al.*, 2007; Vieites *et al.*, 2007, 2009). Substitutions between some highly divergent sequences are likely severely saturated (e.g., Zheng *et al.*, 2011). However, this does not imply that the evolutionary time-order revealed from the analysis is unreliable. In the molecular clock approach, divergence times between organisms relate to numbers of substitutions accumulated in sequences. Saturation due to multiple hits usually results in greater percentage of underestimated substitutions between more divergent sequences (Nei and Kumar, 2000; Arbogast *et al.*, 2002). As proportional time estimates were compared as ratios in this study, the effect of saturation would likely compress the difference between divergence dates, rather than result in a statistically significant order of time.

Poyarkov *et al.* (2012) recently described a new species, *Onychodactylus zhangyapingi*, which may complicate our analysis. *Onychodactylus zhangyapingi* occurs in northeastern China and is the sister group of Japanese congeners (Poyarkov *et al.*, 2012). This relationship suggests that we likely overestimated the relative age of Japanese *Onychodactylus* (O). The reason of the exclusion of this species in our final analysis is that the available data for this new species are far less than those in dataset Data-MC. Nevertheless, the splitting event O between *O. zhaoermii* and (*O. zhangyapingi* + Japanese *Onychodactylus*) and the split between *O. zhangyapingi* and Japanese *Onychodactylus* are close to each other and both are relatively deep (Poyarkov *et al.*, 2012). Therefore, our overestimate is likely insignificant to the age-diversity pattern (Figure 4).

**4.2 Cryptic diversity in Japanese *Hynobius*** In addition to lineages corresponding to the 17 (of 18) species sampled, six other major lineages were identified including Hnae-100, Hyat-1, Hyat-2, Hbou-100, Hneb-8, and Group-II (Figure 2; Supplementary Material 5). The genetic distances between them and other lineages were at least similar to the reference interspecific distances of the genus (Table 1). The two major lineages of *H. naevius*, Hnae-1 and Hnae-100, were not sister lineages and they corresponded to lineages identified by Tominaga *et al.* (2006). Lineage 1 was from northwestern Kyushu Island (Hnae-1) and Lineage 2 from the other populations (Hnae-100). Because the type locality of *H. naevius* was estimated to be in the northwestern Kyushu Island (Tominaga and Matsui, 2007), the other populations may be a cryptic species. Consistent with this finding, these two groups differ by two of 20 allozyme loci (fixed alleles at *sIDH-A* and nearly fixed at *mACOH-A*) and by two-dimensional scaling of allozyme data based on 19 (of 20) polymorphic loci (Tominaga *et al.*, 2005a). Two-dimensional scaling analyses of morphology also separate the lineages (Tominaga *et al.*, 2005b; Tominaga and Matsui, 2008).

Three major lineages occur among samples of *H. yatsui*: the lineage from Kyushu Island, Hyat-2, and Hyat-1, of which the former two are sister-lineages (Figure 2; Supplementary Material 5). The former lineage contains all haplotypes on the island and this includes the type locality of *H. yatsui* (Tominaga and Matsui, 2008). This lineage, named KYUSHU or Kyushu-B, is clearly distinguishable from non-Kyushu populations by allozymes (e.g., loci *PGDH-A* and *PGM-C*), morphology, and matrilineal studies (Tominaga *et al.*, 2005a, b, 2006; Tominaga and Matsui, 2008). The non-Kyushu Island morphological groups and genetic lineages, including Hyat-1 and Hyat-2, suggest cryptic diversity and this requires further study.

Samples of *H. boulengeri* comprise two distantly related lineages. The lineage represented by haplotypes Hbou-1 and Hbou-101 corresponds to the true *H. boulengeri*, as restricted to Honshu Island by Nishikawa *et al.* (2007) based on morphological and allozymic variation. The other lineage, Hbou-100, may represent either an unnamed species (Nishikawa *et al.* 2007) or *H. hirosei*, which was removed from the synonymy of *H. boulengeri* by Nishikawa *et al.* (2007). The precise collecting locality of Hbou-100 remains unknown and this precludes a determination.

*Hynobius nebulosus* has three haplotypes from

near the type locality of Nagasaki: Hneb-G, Hneb-9, and Hneb-100. Other, distantly related samples form two major lineages that are not sister groups: Group-II and Hneb-8 (Figure 2). Lineages represented by the three haplotypes may be sister-groups (S-H test, $P > 0.05$) and the genetic distances between them are less than most values for the reference species (Table 1). Therefore, we follow Matsui *et al.* (2006) in assigning all three haplotypes to *H. nebulosus*. Allozyme analyses for *H. nebulosus sensu lato* identify three candidate cryptic species: eastern, montane, and Chugoku groups (Matsui *et al.*, 2006). Our Group-II corresponds to the eastern group. Because the sampling site (Gobo-shi) of lineage Hneb-8 was not included in the study of Matsui *et al.* (2006), we cannot determine if this is another of their potential cryptic species.

Underestimations of variation may obscure our understanding of evolutionary processes (Olsson *et al.*, 2005; Kaliontzopoulou *et al.*, 2011). Our analyses reinforce the high level of cryptic diversity within Japanese *Hynobius* as revealed in previous morphological and allozymic studies, particularly by the recognition of non-monogenealogical (sensu Murphy and Méndez de la Cruz, 2010; Gao *et al.*, 2012) species. This finding supports the importance of having dense taxon sampling in genealogical and taxonomic studies (e.g., Olsson *et al.*, 2005). A cryptic species is not necessarily the sister group of the species from which it differs (Donald *et al.*, 2005; Bickford *et al.*, 2007; Seifert, 2009), especially for groups such as amphibians that exhibit conserved morphological evolution and extensive homoplasy (Parra-Olea and Wake, 2001; Fouquet *et al.*, 2007).

**4.3 Northern glacial refugium of *Hynobius leechii***

Multiple refugia might have served *H. leechii* during Pleistocene glacial cycling. This species currently occurs on the Korean Peninsula and adjacent northeastern China with a general north-south distribution. Substantial genetic diversity occurs on the southern peninsula yet with a similar sampling area to the southern one, extremely low diversity occurs northwards. The genealogy reveals a southern origin of the northern form (Figure 2). This pattern indicates that the southern peninsula provided a glacial refugium for *H. leechii* (Hewitt, 2000; Provan and Bennett, 2008), and this has been indicated for a variety of organisms (e.g., Kong, 2000; Serizawa *et al.*, 2002; Zhang H. *et al.*, 2008).

For *H. leechii*, another refugium probably existed north of the peninsula. Several observations are consistent with this notion. First, among the three northern haplotypes, Hlee-92, the only haplotype found in most sampling sites (Supplementary Material 1), is widespread throughout the northern sampling area (Figure 3). The two other haplotypes, Hlee-G1 and Hlee-91, differ from the former by one or two nucleotide substitutions only (Figure 3). Their distributions are far more restricted geographically and this pattern infers a local origin. These distributions suggest a postglacial range expansion (Provan and Bennett, 2008). Second, on the network and genealogy, these haplotypes substantially differ from the most closely related interior southern haplotypes, in the case of the network by at least ten unobserved haplotypes involving 11 substitutions. A p-distance of 1.49% based on the 740 sites of *cyt b* separates them. It is unlikely that this level of divergence accumulated during a northwards post-last glacial maximum (LGM) dispersal of this species from the Korean Peninsula refugium (Canestrelli *et al.*, 2006; Provan and Bennett, 2008). Given the broadly used evolutionary rate of 0.64%–1.00% divergence per million years per lineage for vertebrate *cyt b* gene (e.g., Tominaga *et al.*, 2010), these haplotypes should have diverged long before the LGM, which occurred about 0.02 Ma. The northern form probably persisted in situ throughout the LGM. These results agree with the notion that postglacial colonization of high latitude regions can be from local sources within or close to the regions, rather than from the more distant, southern refugium (Stewart and Lister, 2001; Pearson, 2006).

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