

# Taxonomic Validity of *Hynobius Hidamontanus* (Caudata: Hynobiidae): Descriptions of Four New Species from Western Honshu, Japan

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Received September 16, 2023; Revised October 17, 2023; Accepted October 24, 2023

**Abstract** Four new species of *Hynobius* are described from the Chugoku Mountains, Japan. The complex of *Hynobius utsunomiyaorum*, including *Hynobius hidamontanus*, was divided into six groups based on morphological and molecular analyses. The Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, and northeastern Okayama groups of *H. utsunomiyaorum* were described as *Hynobius pseudoutsunomiyaorum* sp. nov., *Hynobius mengamemontanus* sp. nov., *Hynobius sakuhokumontanus* sp. nov., and *Hynobius ushiromontanus* sp. nov., respectively. Molecular analyses of samples from across the entire distribution range of *H. utsunomiyaorum* revealed that *H. utsunomiyaorum* (including *H. hidamontanus*) has six genetic groups. Morphological analyses suggested that males of the four new species are morphologically different from *H. utsunomiyaorum* according to specific characters. The distribution of the new species, as well as those of *H. utsunomiyaorum* and *H. hidamontanus*, were determined and compared to indicate the isolation among the species. Although further sampling from the northern part of Okayama Prefecture is needed to determine the distribution ranges of *H. utsunomiyaorum* and *H. sakuhokumontanus* sp. nov., our results suggest that the distribution area of true *H. utsunomiyaorum* is mainly limited to the central area of the Chugoku Mountains.

**Keywords:** Chugoku Mountains, conservation, cryptic species, geographic isolation, Hokuriku District, mitochondrial DNA

**Cite This Article:** Hiroataka Sugawara, Takayuki Iwata, Jun-ichi Naito, Masaru Yamada, Kazuto Onomura, and Masahiro Nagano, "Taxonomic Validity of *Hynobius Hidamontanus* (Caudata: Hynobiidae): Descriptions of Four New Species from Western Honshu, Japan." American Journal of Zoological Research, vol. 8, no. 1 (2023): 6-26. doi: 10.12691/ajzr-8-1-2.

## 1. Introduction

The genus *Hynobius*, is the most diversified within the family Hynobiidae with 66 described species and is found in eastern Asia [1]. Most species in the genus are found in Japan, with 48 species distributed in throughout the country (48/66 = 72.7%) [1]. Traditionally, *Hynobius* species have been identified based on morphological characters [2,3]. However, *Hynobius* exhibits high number of cryptic species, making identification without molecular markers and collection site information difficult [4]. Molecular markers have revealed the existence of

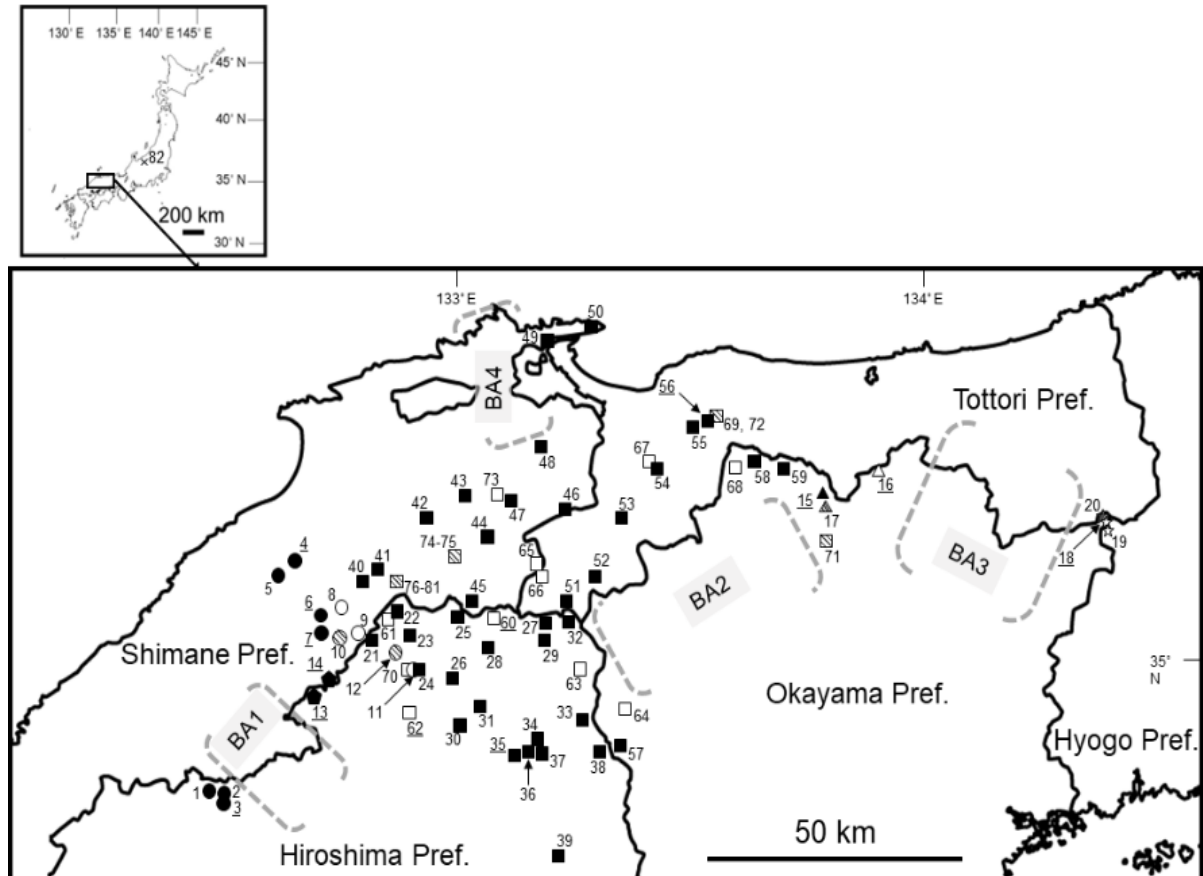
cryptic species in *Hynobius* [5,6], and they are essential for classifying this genus and describing new species.

The Hiba salamander, *Hynobius utsunomiyaorum*, was described from Rokunohara, Mt. Hiba, Saijocho Yuki, Shobara City, Hiroshima Prefecture, and is found in Hyogo, Okayama, Hiroshima, Tottori, and Shimane Prefectures, Chugoku District, Japan [7]. The Hakuba salamander, *Hynobius hidamontanus*, was described from Ochikura Swamp, Hokujo, Hakuba Village, Nagano Prefecture, and is found in Niigata, Toyama, and Gifu Prefectures [8,9]. *Hynobius utsunomiyaorum* has four distinct groups with *H. hidamontanus* distributed around the Hokuriku District [7]. The monophyly of the clade, including *H. utsunomiyaorum* and *H. hidamontanus*, was

strongly supported by mitochondrial DNA analysis results [maximum likelihood (ML) estimation: bootstrap value = 99%; Bayesian inference (BI): posterior probability = 1.00] [7]. However, the results of molecular analyses using more samples suggested that the monophyly is doubtful according to BI [10]. Thus, based on the results of previous studies [7,10] and the phylogenetic species concept, *H. hidamontanus* may be an invalid species because it is clearly included in the clade of *H. utsunomiyaorum*. Moreover, if *H. hidamontanus* is a valid species according to the phylogenetic species concept, *H. utsunomiyaorum* should be divided into at least three species based on the data of previous study [7]: the Shimane (haplotype H9-H11), northern Okayama (haplotype H6-H8), and Hiroshima (haplotype H1-H5) groups (haplotype numbers follow those of the study [7]). In addition, the northern Okayama group might be divisible into two groups, the northern central Okayama (haplotype H6) and northeastern Okayama (haplotype H7-H8) groups, although these are monophyletic groups [7]. Matsui et al. [7] performed molecular analysis using only 18 individuals from eleven localities, and Sugawara et al. [10] did not collect samples from the entire distribution range of *H. utsunomiyaorum*. Thus, the phylogenetic relationship of *H. utsunomiyaorum* and *H. hidamontanus* should be reassessed using samples collected across the entire distribution range of *H. utsunomiyaorum*. From a morphological perspective, the commonality of the four groups (Shimane, northern central Okayama, northeastern Okayama, and Hiroshima groups) of *H. utsunomiyaorum*

is unreliable because Matsui et al. [7] did not compare these groups. Although *H. hidamontanus* is included in the *H. utsunomiyaorum* clade, Matsui et al. [7] and Sugawara et al. [10] did not use *H. hidamontanus* in their morphological analyses; thus, the morphologies of the four groups of *H. utsunomiyaorum* and *H. hidamontanus* must also be compared. Additionally, *H. utsunomiyaorum* has a wide distribution range, so there is a need to determine whether another genetically distinct group exists. If such a group is found, it should be included in subsequent morphological analyses.

Frost and Hillis [11] recommend using phylogenetic and evolutionary species concepts in herpetology. Additionally, Dubois [12] mentioned that essential features must be directly derived from characteristics observed in specimens rather than indirectly inferred when a new species is described. The original description of *Hynobius kunibiki* before being included in *H. utsunomiyaorum* used three species concepts (i.e. phylogenetic, evolutionary, and morphological species concepts) to support their conceptions [10]. Thus, we followed these three species concepts consistent with previous studies [4,10]. First, we determined the number of candidate species included in *H. utsunomiyaorum* based on the phylogenetic and evolutionary species concepts. Second, we evaluated the species validity of each candidate species and *H. hidamontanus* based on the morphological species concept. Finally, we described several new species and revealed the distribution ranges of these species in detail based on the results of our study.



**Figure 1.** Sampling map of this study. Additional explanations are described in the Materials and Methods section.

## 2. Materials and Methods

### 2.1. Molecular Survey

We sampled 49 individuals from 49 localities from April 2007 to June 2022 (Table 1; Figure 1) for phylogenetic analyses. Figure 1 indicates the sampling map of *Hynobius* species, according to the present study. The cross in the upper left area shows the type locality of *H. hidamontanus* (Figure 1). The enlarged area includes the central Chugoku District where the *H. utsunomiyaorum* complex is found (Figure 1). The closed symbols correspond to each of four groups sequenced in this study: Mt. Sanbe and Kitahiroshima group (closed circles), Mt. Mengame group (closed pentagons), northern central Okayama group (closed triangle), and widely distributed group (closed squares) (Figure 1). The open symbols correspond to each of four groups sequenced by Matsui et al. [7] or Sugawara et al. [10]: Mt. Sanbe and Kitahiroshima group (open circles), northern central Okayama group (open triangle), northeastern Okayama

group (open stars), and widely distributed group (open squares) (Figure 1). The open symbols including diagonal lines correspond to each of four groups described by Matsui et al. [7] or Hayashi and Ooi [13] for which the detailed locality is unclear: Mt. Sanbe and Kitahiroshima group (shaded circles), northern central Okayama group (shaded triangle), northeastern Okayama group (shaded star), and widely distributed group (shaded squares) (Figure 1). Dotted lines including BA (1-4) represent the blank area of distribution of the *H. utsunomiyaorum* complex (Figure 1). Table 1 shows the sample list of *Hynobius* species using this study. Population number corresponds to the localities in Figure 1 (Table 1). Asterisks after sampling localities show the type locality of each species (Table 1). In this study, the widely distributed group means the true *H. utsunomiyaorum* (sensu stricto), and four groups have another species name as follows: Mt. Sanbe and Kitahiroshima group = *Hynobius pseudoutsunomiyaorum* sp. nov.; Mt. Mengame group = *Hynobius mengamemontanus* sp. nov.; northern central Okayama group = *Hynobius sakuhokumontanus* sp. nov.; northeastern Okayama group = *Hynobius ushiromontanus* sp. nov.

Table 1. Sample list using molecular analyses

| Population | Species                                       | Sampling locality                                                 | Accession no.<br>(cytochrome b) | Label in Figure 2 |
|------------|-----------------------------------------------|-------------------------------------------------------------------|---------------------------------|-------------------|
| 1          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Oasa, Kitahiroshima Town, Hiroshima Prefecture                    | LC746025                        | PSE01             |
| 2          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Miyazako, Kitahiroshima Town, Hiroshima Prefecture                | LC746026                        | PSE02             |
| 3          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Shinjo, Kitahiroshima Town, Hiroshima Prefecture*                 | LC746027                        | PSE03             |
| 4          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Yamaguchicho Yamaguchi, Oda City, Shimane Prefecture              | LC746028                        | PSE04             |
| 5          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Sanbecho Koyahara, Oda City, Shimane Prefecture                   | LC746029                        | PSE05             |
| 6          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Shimokijima, Iinan Town, Shimane Prefecture                       | LC746030                        | PSE06             |
| 7          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Sakedani, Misato Town, Shimane Prefecture                         | LC746031                        | PSE07             |
| 8          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Nagatani, Iinan Town, Shimane Prefecture                          | LC605112                        | Utsunomiyaorum07  |
| 9          | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Oda, Iinan Town, Shimane Prefecture                               | LC605113                        | Utsunomiyaorum08  |
| 10         | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Iinan Town, Shimane Prefecture                                    | LC436374                        | H9                |
| 11         | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Kanaotoge, Takanochi Okumonde, Shobara City, Hiroshima Prefecture | LC436375                        | H10               |
| 12         | <i>Hynobius pseudoutsunomiyaorum</i> sp. nov. | Shobara City, Hiroshima Prefecture                                | LC436376                        | H11               |
| 13         | <i>Hynobius mengamemontanus</i> sp. nov.      | Sakugicho Okamibuchi, Miyoshi City, Hiroshima Prefecture*         | LC746032                        | MEN01             |
| 14         | <i>Hynobius mengamemontanus</i> sp. nov.      | Kamiakana, Iinan Town, Shimane Prefecture                         | LC746033                        | MEN02             |
| 15         | <i>Hynobius sakuhokumontanus</i> sp. nov.     | Hiruzenshitao, Maniwa City, Okayama Prefecture                    | LC746034                        | SAK01             |
| 16         | <i>Hynobius sakuhokumontanus</i> sp. nov.     | Kamisaibara, Kagamino Town, Okayama Prefecture*                   | LC605117                        | Utsunomiyaorum12  |
| 17         | <i>Hynobius sakuhokumontanus</i> sp. nov.     | Maniwa City, Okayama Prefecture                                   | LC436371                        | H6                |
| 18         | <i>Hynobius ushiromontanus</i> sp. nov.       | Ogaya, Nishiawakura Village, Okayama Prefecture*                  | LC605118                        | Utsunomiyaorum13  |
| 19         | <i>Hynobius ushiromontanus</i> sp. nov.       | Chikusacho Nishigochi, Shiso City, Hyogo Prefecture               | LC436372                        | H7                |
| 20         | <i>Hynobius ushiromontanus</i> sp. nov.       | Nishiawakura Village, Okayama Prefecture                          | LC436373                        | H8                |
| 21         | <i>Hynobius utsunomiyaorum</i>                | Takanochi Kobo, Shobara City, Hiroshima Prefecture                | LC746035                        | UTS01             |
| 22         | <i>Hynobius utsunomiyaorum</i>                | Takanochi Wananbara, Shobara City, Hiroshima Prefecture           | LC746036                        | UTS02             |
| 23         | <i>Hynobius utsunomiyaorum</i>                | Takanochi Shinichi, Shobara City, Hiroshima Prefecture            | LC746037                        | UTS03             |
| 24         | <i>Hynobius utsunomiyaorum</i>                | Takanochi Okumonde, Shobara City, Hiroshima Prefecture            | LC746038                        | UTS04             |
| 25         | <i>Hynobius utsunomiyaorum</i>                | Hiwacho Moriwaki, Shobara City, Hiroshima Prefecture              | LC746039                        | UTS05             |
| 26         | <i>Hynobius utsunomiyaorum</i>                | Hiwacho Koyabara, Shobara City, Hiroshima Prefecture              | LC746040                        | UTS06             |

| Population | Species                        | Sampling locality                                              | Accession no.<br>(cytochrome b) | Label in <a href="#">Figure 2</a> |
|------------|--------------------------------|----------------------------------------------------------------|---------------------------------|-----------------------------------|
| 27         | <i>Hynobius utsunomiyaorum</i> | Saijocho Misaka, Shobara City, Hiroshima Prefecture            | LC746041                        | UTS07                             |
| 28         | <i>Hynobius utsunomiyaorum</i> | Saijocho Kumano, Shobara City, Hiroshima Prefecture            | LC746042                        | UTS08                             |
| 29         | <i>Hynobius utsunomiyaorum</i> | Saijocho Hitotobara, Shobara City, Hiroshima Prefecture        | LC746043                        | UTS09                             |
| 30         | <i>Hynobius utsunomiyaorum</i> | Kawatecho, Shobara City, Hiroshima Prefecture                  | LC746044                        | UTS10                             |
| 31         | <i>Hynobius utsunomiyaorum</i> | Kawagitacho, Shobara City, Hiroshima Prefecture                | LC746045                        | UTS11                             |
| 32         | <i>Hynobius utsunomiyaorum</i> | Tojocho Onuka, Shobara City, Hiroshima Prefecture              | LC746046                        | UTS12                             |
| 33         | <i>Hynobius utsunomiyaorum</i> | Tojocho Kawahigashi, Shobara City, Hiroshima Prefecture        | LC746047                        | UTS13                             |
| 34         | <i>Hynobius utsunomiyaorum</i> | Tojocho Taishakumido, Shobara City, Hiroshima Prefecture       | LC746048                        | UTS14                             |
| 35         | <i>Hynobius utsunomiyaorum</i> | Soryocho Goka, Shobara City, Hiroshima Prefecture              | LC746049                        | UTS15                             |
| 36         | <i>Hynobius utsunomiyaorum</i> | Furukawa, Jinsekikogen Town, Hiroshima Prefecture              | LC746050                        | UTS16                             |
| 37         | <i>Hynobius utsunomiyaorum</i> | Aido, Jinsekikogen Town, Hiroshima Prefecture                  | LC746051                        | UTS17                             |
| 38         | <i>Hynobius utsunomiyaorum</i> | Ono, Jinsekikogen Town, Hiroshima Prefecture                   | LC746052                        | UTS18                             |
| 39         | <i>Hynobius utsunomiyaorum</i> | Chichikino, Jinsekikogen Town, Hiroshima Prefecture            | LC746053                        | UTS19                             |
| 40         | <i>Hynobius utsunomiyaorum</i> | Tsugaka, Iinan Town, Shimane Prefecture                        | LC746054                        | UTS20                             |
| 41         | <i>Hynobius utsunomiyaorum</i> | Yoshidacho Yoshida, Unnan City, Shimane Prefecture             | LC746055                        | UTS21                             |
| 42         | <i>Hynobius utsunomiyaorum</i> | Kisukicho Yumura, Unnan City, Shimane Prefecture               | LC746056                        | UTS22                             |
| 43         | <i>Hynobius utsunomiyaorum</i> | Daitocho Shinobuchi, Unnan City, Shimane Prefecture            | LC746057                        | UTS23                             |
| 44         | <i>Hynobius utsunomiyaorum</i> | Kamedake, Okuizumo Town, Shimane Prefecture                    | LC746058                        | UTS24                             |
| 45         | <i>Hynobius utsunomiyaorum</i> | Omaki, Okuizumo Town, Shimane Prefecture                       | LC746059                        | UTS25                             |
| 46         | <i>Hynobius utsunomiyaorum</i> | Hakutacho Kusano, Yasugi City, Shimane Prefecture              | LC746060                        | UTS26                             |
| 47         | <i>Hynobius utsunomiyaorum</i> | Hirosecho Nishidani, Yasugi City, Shimane Prefecture           | LC746061                        | UTS27                             |
| 48         | <i>Hynobius utsunomiyaorum</i> | Hirosecho Toda, Yasugi City, Shimane Prefecture                | LC746062                        | UTS28                             |
| 49         | <i>Hynobius utsunomiyaorum</i> | Mihonosekicho Moriyama, Matsue City, Shimane Prefecture        | LC746063                        | UTS29                             |
| 50         | <i>Hynobius utsunomiyaorum</i> | Mihonosekicho Mihonoseki, Matsue City, Shimane Prefecture      | LC746064                        | UTS30                             |
| 51         | <i>Hynobius utsunomiyaorum</i> | Yukawa, Nichinan Town, Tottori Prefecture                      | LC746065                        | UTS31                             |
| 52         | <i>Hynobius utsunomiyaorum</i> | Fukuzuka, Nichinan Town, Tottori Prefecture                    | LC746066                        | UTS32                             |
| 53         | <i>Hynobius utsunomiyaorum</i> | Kusumi, Hino Town, Tottori Prefecture                          | LC746067                        | UTS33                             |
| 54         | <i>Hynobius utsunomiyaorum</i> | Sanbu, Hoki Town, Tottori Prefecture                           | LC746068                        | UTS34                             |
| 55         | <i>Hynobius utsunomiyaorum</i> | Kobayashi, Hoki Town, Tottori Prefecture                       | LC746069                        | UTS35                             |
| 56         | <i>Hynobius utsunomiyaorum</i> | Daisen, Daisen Town, Tottori Prefecture                        | LC746070                        | UTS36                             |
| 57         | <i>Hynobius utsunomiyaorum</i> | Bicchuchō Nishiyama, Takahashi City, Okayama Prefecture        | LC746071                        | UTS37                             |
| 58         | <i>Hynobius utsunomiyaorum</i> | Hiruzenkamifukuda, Maniwa City, Okayama Prefecture             | LC746072                        | UTS38                             |
| 59         | <i>Hynobius utsunomiyaorum</i> | Hiruzenshimofukuda, Maniwa City, Okayama Prefecture            | LC746073                        | UTS39                             |
| 60         | <i>Hynobius utsunomiyaorum</i> | Rokunohara, Saijocho Yuki, Shobara City, Hiroshima Prefecture* | LC605106                        | Utsunomiyaorum01                  |
| 61         | <i>Hynobius utsunomiyaorum</i> | Takanochō Agarihara, Shobara City, Hiroshima Prefecture        | LC605107                        | Utsunomiyaorum02                  |
| 62         | <i>Hynobius utsunomiyaorum</i> | Kuchiwacho Mukoizumi, Shobara City, Hiroshima Prefecture       | LC605108                        | Utsunomiyaorum03                  |
| 63         | <i>Hynobius utsunomiyaorum</i> | Tojocho Utsubori, Shobara City, Hiroshima Prefecture           | LC605109                        | Utsunomiyaorum04                  |
| 64         | <i>Hynobius utsunomiyaorum</i> | Tettacho Tabuchi, Niimi City, Okayama Prefecture               | LC605110                        | Utsunomiyaorum05                  |
| 65         | <i>Hynobius utsunomiyaorum</i> | Takezaki, Okuizumo Town, Shimane Prefecture                    | LC605111                        | Utsunomiyaorum06                  |
| 66         | <i>Hynobius utsunomiyaorum</i> | Kamihagiyama, Nichinan Town, Tottori Prefecture                | LC605114                        | Utsunomiyaorum09                  |
| 67         | <i>Hynobius utsunomiyaorum</i> | Ikeno, Nanbu Town, Tottori Prefecture                          | LC605115                        | Utsunomiyaorum10                  |
| 68         | <i>Hynobius utsunomiyaorum</i> | Hiruzenkamitokuyama, Maniwa City, Okayama Prefecture           | LC605116                        | Utsunomiyaorum11                  |
| 69         | <i>Hynobius utsunomiyaorum</i> | Daisen Town, Tottori Prefecture                                | LC436443                        | H1                                |
| 70         | <i>Hynobius utsunomiyaorum</i> | Kanaotoge, Takanochō Okumonde, Shobara City,                   | LC436367                        | H2                                |

| Population           | Species                          | Sampling locality                                         | Accession no.<br>(cytochrome b) | Label in Figure 2   |
|----------------------|----------------------------------|-----------------------------------------------------------|---------------------------------|---------------------|
| Hiroshima Prefecture |                                  |                                                           |                                 |                     |
| 71                   | <i>Hynobius utsunomiyaorum</i>   | Kagamino Town, Okayama Prefecture                         | LC436368                        | H3                  |
| 72                   | <i>Hynobius utsunomiyaorum</i>   | Daisen Town, Tottori Prefecture                           | LC436369                        | H4                  |
| 73                   | <i>Hynobius utsunomiyaorum</i>   | Hirosecho Okutawara, Yasugi City, Shimane Prefecture      | LC436370                        | H5                  |
| 74                   | <i>Hynobius utsunomiyaorum</i>   | Okuizumo Town (former Yokota Town), Shimane Prefecture    | LC518948                        | SSN049              |
| 75                   | <i>Hynobius utsunomiyaorum</i>   | Okuizumo Town (former Yokota Town), Shimane Prefecture    | LC518949                        | SSN050              |
| 76                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518950                        | SSN051              |
| 77                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518951                        | SSN052              |
| 78                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518952                        | SSN053              |
| 79                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518953                        | SSN054              |
| 80                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518954                        | SSN055              |
| 81                   | <i>Hynobius utsunomiyaorum</i>   | Unnan City (former Yoshida Village), Shimane Prefecture   | LC518955                        | SSN056              |
| 82                   | <i>Hynobius hidamontanus</i>     | Hokujo, Hakuba Village, Nagano Prefecture*                | LC225434                        | H. hidamontanus     |
|                      | <i>Hynobius abuensis</i>         | Atokaneshimo, Yamaguchi City, Yamaguchi Prefecture*       | LC605122                        | H. abuensis         |
|                      | <i>Hynobius akiensis</i>         | Mirasakacho Haizuka, Miyoshi City, Hiroshima Prefecture*  | LC436384                        | H. akiensis         |
|                      | <i>Hynobius geiyoensis</i>       | Nakano, Osakikamijima Town, Hiroshima Prefecture*         | LC647835                        | H. geiyoensis       |
|                      | <i>Hynobius kunibiki</i>         | Nishinomura, Kamionochi, Matsue City, Shimane Prefecture* | LC605055                        | H. kunibiki         |
|                      | <i>Hynobius sumidai</i>          | Tamaricho, Takehara City, Hiroshima Prefecture*           | LC647839                        | H. sumidai          |
|                      | <i>Hynobius tosashimizuensis</i> | Tosashimizu City, Kochi Prefecture*                       | LC436447                        | H. tosashimizuensis |

Total genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The mitochondrial cytochrome b gene (630-base-pairs) was amplified using ExTaq (TaKaRa, Tokyo, Japan) with primers L14010 (5'-TAHGGWGAHGGATTWGAWGCMAWGC-3') and H14778 (5'-AARTAYGGGTGRAADGRRAYTTTTRCT-3') [14]. The PCR reaction mix (total volume: 10 µl) contained 1.0 µl of 10×Ex Taq Buffer, 0.8 µl of 25 mM dNTP mix, 0.5 µl of each of the forward and reverse primers (10 pM), 0.05 µl of Taq polymerase, 6.15 µl of distilled deionized water, and 1.0 µl of template DNA. The PCR protocols implemented using a T100™ thermal cycler (Bio-Rad, Hercules, CA, USA) were as follows: an initial 3-min denaturing step at 94°C; 40 cycles of 30 s at 94°C, 45 s at 56°C, and 90 s at 72°C; with a final 10-min extension at 72°C. The PCR products were purified with illustra™ Exo ProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) and sequenced using BigDye® Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Acquired DNA sequences were deposited to the DNA Data Bank of Japan (Table 1), and these sequences were aligned using MEGA X [15]. Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) estimation with closely related species, including the highland and Aki groups [7], and *Hynobius abuensis* and *Hynobius tosashimizuensis* as the outgroup, given that they are the closest species of the highland and Aki groups [7]. We estimated the best-fit nucleotide substitution model based on the Bayesian information criterion (BIC) [16] and corrected Akaike's information criterion (AICc) [17] using jModelTest 2 [18]. We selected the Hasegawa-Kishino-Yano (HKY) model with gamma distribution in BI and ML. We constructed Bayesian and maximum likelihood trees using MrBayes 3.2 [19] and MEGA X [15], respectively. For Bayesian analyses, we performed

two independent MCMC runs for 2000000 generations; with a sample frequency of 100. In Bayesian analysis, we examined the stationarity of the likelihood scores of sampled trees using Tracer version 1.7 (<http://tree.bio.ed.ac.uk/software/tracer/>), and the first 25% of generations were discarded as burn-in. The assessment of monophyly was performed by values of posterior probability (PP) and bootstrap (BS) based on the criteria of Huelsenbeck and Rannala [20] and Hillis and Bull [21]: monophyletic group = PP ≥ 0.95 and BP ≥ 70.

## 2.2. Morphological Survey

Underlined localities in Figure 1 show the sampling points of individuals used in morphological comparisons. In this study, we used only male individuals for morphological analyses, consistent with Matsui et al. [7], because we could not collect sufficient female specimens to support statistical analyses, and the morphological differences between the sexes of *H. utsunomiyaorum* are limited [10]. We sampled 67 males of *H. utsunomiyaorum* from April 2019 to May 2022: Mt. Sanbe and Kitahiroshima group, 14 individuals from four populations (Pops. 3, 4, 6, and 7); Mt. Mengame group, 10 individuals from two populations (Pops. 13 and 14); northern central Okayama group, 14 individuals from two populations (Pops. 15 and 16); northeastern Okayama group, 14 individuals from one population (Pop. 18); and widely distributed group, 15 individuals from four populations (Pops. 35, 56, 60, and 62) (Table 1; Figure 1). We also sampled seven males of *H. hidamontanus* in April 2021 at the type locality of this species (Table 1; Figure 1) after obtaining permission from the governments of Nagano Prefecture and Hakuba Village. The collected individuals were measured under anesthesia using ethyl

3-aminobenzoate methanesulfonate salt (Sigma-Aldrich®, St. Louis, MO, USA) diluted 1000 times with water [22]. From a conservation perspective, measured individuals were returned to their site of capture except for the candidate individuals of type specimens or candidate individuals required for redescribing *H. utsunomiyaorum*. Before the individuals were returned, photos were taken of the dorsal, ventral, and lateral sides on a black back-ground, and tissue samples (preserved in 99.9% ethanol) were collected from the tail tips of all individuals as evidence of collection. For all examined individuals, a vernier caliper was used to take 21 measurements following Nishikawa et al. [23]: snout-vent length (SVL), trunk length (TRL), axilla-groin distance (AGD), head length (HL), tail length (TAL), median tail width (MTAW), median tail height (MTAH), vomerine teeth length (VTL), and vomerine teeth wide (VTW), head width (HW), forelimb length (FLL), hindlimb length (HLL), second finger length (2FL), third finger length (3FL), third toe length (3TL), internarial distance (IND), interorbital distance (IOD), upper eyelid length (UEL), snout length (SL), upper eyelid width (UEW), lower jaw length (LJL). We also checked the presence of mid-dorsal line between forelimb and hindlimb (MDL), the presence of distinct white spots on ventral side of the body (DWSV), the presence of distinct white spots on lateral sides of the body (DWSL), the presence of distinct yellowish-brown lines on the dorsal (DYBLDT) and ventral (DYBLVT) sides of the tail, and presence of distinct gular mottling (DGM) for each individual. The number of costal folds between the adpressed limbs (CFBALN) and the number of costal grooves (CGN) were counted. The counting method of CGN was followed by Matsui et al. [7]. For assessments of the morphological differences among species, we performed the statistical approaches using R [24] with significance level equal 0.05. Before the statistical tests, we tested for normality using Shapiro-Wilk test. When data did not follow a normal distribution and variances among populations were not equal, we performed Steel-Dwass tests. On the other hand, when data followed a normal distribution, we tested for homoscedasticity using Bartlett's test. As the next step, when the variances among the populations were equal, we performed Tukey-Kramer tests, but when variances were not equal, we performed Games-Howel tests. In addition, we performed canonical discriminant analysis using standardized values for the 20 measurements and SVL to examine the overall morphological variation among six groups (including *H. hidamontanus*).

### 2.3. Measurement of Type Specimens

When the measurement of holotype and topotype specimens, we took 43 measurements referred to Nishikawa et al. [23]: SVL, TRL, AGD, HL, TAL, MTAW, MTAH, basal tail width (BTAW), basal tail height (BTAH), left vomerine teeth length (LVTL), right vomerine teeth length (RVTL), VTW, HW, left forelimb length (LFLL), left hindlimb length (LHLL),

right forelimb length (RFLL), right hindlimb length (RHLL), left first finger length (L1FL), left second finger length (L2FL), left third finger length (L3FL), left fourth finger length (L4FL), right first finger length (R1FL), right second finger length (R2FL), right third finger length (R3FL), right fourth finger length (R4FL), left first toe length (L1TL), left second toe length (L2TL), left third toe length (L3TL), left fourth toe length (L4TL), right first toe length (R1TL), right second toe length (R2TL), right third toe length (R3TL), right fourth toe length (R4TL), IND, IOD, left upper eyelid length (LUEL), right upper eyelid length (RUEL), left snout length (LSL), right snout length (RSL), left upper eyelid width (LUEW), right upper eyelid width (RUEW), left lower jaw length (LLJL), and right lower jaw length (RLJL). All measurements and observations of holotype and topotype were carried out after fixation in 10% formalin and transferred to 70% ethanol. To avoid the over-collection of new species, details of type specimens are available only by contacting the museum that stored each specimen.

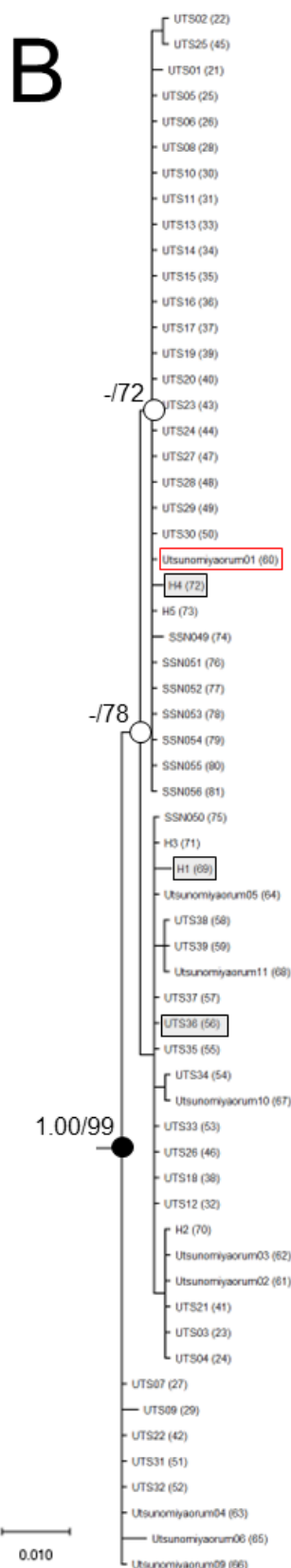
## 3. Results

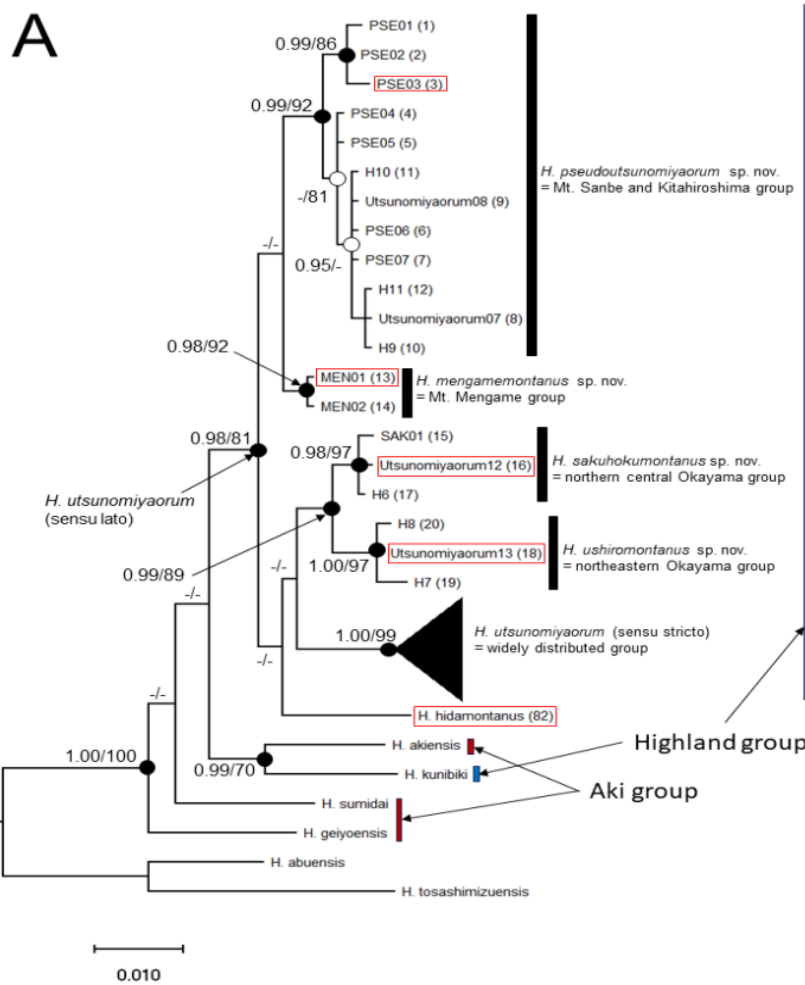
### 3.1. Molecular Survey

Molecular phylogenetic trees based on BI and ML were nearly identical. Scale bars in Figure 2A and 2B show the genetic distance (expected changes per site). Numbers located near the nodes are posterior probabilities (PP) for Bayesian inference and bootstrap values (BS) for maximum likelihood estimation (Figures 2A and 2B). The nodes covered by closed circles and open circles indicate the support by both values or either value, respectively (Figures 2A and 2B). Nodes not covered by circles indicate that both values do not satisfy the conditions of statistical support ( $PP \geq 0.95$  and  $BS \geq 70\%$ ) (Figures 2A and 2B). Values in parentheses after haplotype names correspond to population numbers, as indicated in Table 1 and Figure 1 (Figures 2A and 2B). Labels boxed by red lines show the type locality of six species (Figures 2A and 2B). Labels covered by shaded boxes show the Daisen type of *H. utsunomiyaorum* (Figure 2B). The monophyly of the highland and Aki groups suggested by Matsui et al. [7] was strongly supported by BI and ML, as in the previous study (Figure 2A). The monophyly of the *H. utsunomiyaorum* clade with *H. hidamontanus* was supported by PP and BS, consistent with Matsui et al. [7], and five monophyletic groups of *H. utsunomiyaorum* were confirmed in the clade (Figure 2): Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, northeastern Okayama, and widely distributed (*H. utsunomiyaorum* sensu stricto) groups. The monophyly of the northern central Okayama and northeastern Okayama groups was supported by BI and ML (Figure 2A). In the phylogeny of *H. utsunomiyaorum* sensu stricto, the monophyly of the Daisen type of *H. utsunomiyaorum* lacked support from BI and ML (Figure 2B).

### 3.2. Morphological Survey

Morphological measurements of the five groups and *H. hidamontanus* are presented in Table 2. Specifically, PSE, MEN, SAK, USH, and UTS correspond to the Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, northeastern Okayama, and widely distributed groups, respectively, with HID representing *H. hidamontanus*. Ranges are shown in parentheses (Table 2). The significant values of the measurements among these groups and *H. hidamontanus* are shown in Table 3. In the same way as Table 2, PSE, MEN, SAK, USH, UTS, and HID correspond to the Mt. Sanbe and Kitahiroshima group, Mt. Mengame group, northern central Okayama group, northeastern Okayama group, widely distributed group, and *H. hidamontanus*, respectively. Significant differences at  $P < 0.001$  are shown in bold (Table 3). Definitions of morphological characters are described in the Materials and Methods section. Males showed no significant differences in 21 morphological characters in the following group comparisons: Mt. Sanbe and Kitahiroshima vs. Mt. Mengame, Mt. Sanbe and Kitahiroshima vs. northern central Okayama, Mt. Sanbe and Kitahiroshima vs. widely distributed, and Mt. Mengame vs. northern central Okayama (Table 3). A comparison of males between the Mt. Sanbe and Kitahiroshima and northeastern Okayama groups revealed significant differences in four morphological characters (Table 3). Further, a comparison of males between the Mt. Sanbe and Kitahiroshima group and *H. hidamontanus* showed significant differences in five morphological characters (Table 3). In a comparison between the Mt. Mengame and northeastern Okayama groups, males differed significantly in four morphological characters (Table 3), whereas in a comparison between the Mt. Mengame and widely distributed groups, males differed significantly in only one morphological character (Table 3). Notably, a comparison of males between the Mt. Mengame group and *H. hidamontanus* revealed significant differences in three morphological characters (Table 3). Compared with the northeastern Okayama group, widely distributed group, and *H. hidamontanus*, males from the northern central Okayama differed significantly in ten, two, and three morphological characters, respectively (Table 3). Similarly, compared with the widely distributed groups and *H. hidamontanus*, males from the north eastern Okayama differed significantly in three and ten morphological characters, respectively (Table 3). Finally, comparison of males between the widely distributed group and *H. hidamontanus* showed significant differences in six morphological characters (Table 3).





**Figure 2.** Phylogenetic trees using BI: (A) all species using this study; (B) only true *Hynobius utsunomiyaorum*

**Table 2.** Measurement (mm) of SVL and character ratios (R = %SVL) of TRL to L.JL of six *Hynobius* species

| Trait | PSE<br><i>n</i> = 14     | MEN<br><i>n</i> = 10      | SAK<br><i>n</i> = 14     | USH<br><i>n</i> = 14     | UTS<br><i>n</i> = 15     | HID<br><i>n</i> = 7      |
|-------|--------------------------|---------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| SVL   | 52.6±4.04<br>(46.1-61.8) | 52.4±3.84<br>(46.2-57.8)  | 50.4±2.88<br>(45.5-56.3) | 55.5±3.00<br>(48.7-60.1) | 54.7±5.16<br>(45.1-64.3) | 47.9±2.83<br>(43.3-50.4) |
| TRGL  | 76.7±1.12<br>(75.4-79.1) | 76.5±0.99<br>(74.4-77.8)  | 75.7±0.95<br>(73.9-77.1) | 76.5±2.17<br>(69.8-78.5) | 76.7±0.98<br>(74.8-77.9) | 76.2±0.67<br>(75.0-77.0) |
| RAGD  | 51.6±2.17<br>(48.5-56.2) | 53.3±1.44<br>(51.5-55.3)  | 51.9±2.09<br>(48.3-54.9) | 51.6±1.72<br>(46.6-52.9) | 53.0±1.44<br>(50.6-55.8) | 52.9±1.12<br>(51.3-54.8) |
| RHL   | 24.6±1.26<br>(21.5-26.0) | 23.9±0.67<br>(22.9-24.8)  | 24.8±0.77<br>(23.8-26.1) | 23.6±0.68<br>(22.0-24.8) | 24.0±0.77<br>(23.0-25.6) | 24.6±0.66<br>(23.7-25.2) |
| RTAL  | 70.1±7.49<br>(54.1-80.6) | 70.8±11.73<br>(42.0-83.0) | 78.0±6.70<br>(65.2-86.7) | 72.7±5.32<br>(62.2-80.3) | 71.6±6.62<br>(57.9-82.3) | 79.8±5.63<br>(71.5-89.9) |
| RMTAW | 6.6±1.11<br>(4.7-8.5)    | 7.6±0.91<br>(6.8-9.9)     | 7.4±0.67<br>(6.5-8.7)    | 6.0±0.63<br>(5.0-7.2)    | 6.7±0.79<br>(5.5-8.1)    | 7.7±1.12<br>(5.8-8.7)    |
| RMTAH | 9.9±1.77<br>(6.1-12.4)   | 11.6±0.80<br>(10.4-12.6)  | 10.8±0.85<br>(8.7-11.8)  | 8.8±0.75<br>(7.5-10.3)   | 10.4±1.01<br>(8.3-11.6)  | 12.4±1.24<br>(10.9-13.5) |
| RVTL  | 4.6±0.49<br>(3.7-5.3)    | 4.3±0.40<br>(3.6-4.8)     | 4.3±0.30<br>(3.9-4.8)    | 4.7±0.44<br>(4.0-5.5)    | 4.8±0.50<br>(3.9-5.8)    | 3.9±0.43<br>(3.2-4.3)    |
| RVTW  | 5.6±0.41<br>(4.8-6.3)    | 5.4±0.39<br>(4.9-6.1)     | 5.4±0.55<br>(4.6-6.7)    | 5.3±0.27<br>(4.7-5.7)    | 5.5±0.50<br>(4.5-6.7)    | 5.7±0.34<br>(5.3-6.2)    |
| RHW   | 17.4±0.95<br>(15.5-19.1) | 17.5±0.30<br>(17.1-18.0)  | 17.1±0.55<br>(16.0-17.8) | 16.5±0.58<br>(15.4-17.6) | 16.9±0.77<br>(15.1-18.0) | 18.3±0.65<br>(17.5-19.6) |
| RFL   | 25.8±1.78<br>(23.1-28.6) | 25.1±1.42<br>(23.2-28.1)  | 26.6±1.40<br>(24.3-28.7) | 24.4±1.48<br>(21.8-27.0) | 25.5±1.64<br>(21.8-27.7) | 28.0±0.45<br>(27.4-28.6) |
| RHLL  | 32.3±2.11<br>(28.7-36.5) | 30.2±2.15<br>(26.3-34.4)  | 32.2±1.41<br>(29.9-34.7) | 30.7±1.31<br>(29.1-33.0) | 32.4±1.65<br>(30.5-37.0) | 31.5±0.60<br>(30.7-32.4) |
| R2FL  | 5.1±0.57<br>(4.3-6.1)    | 5.2±0.28<br>(4.8-5.6)     | 5.3±0.44<br>(4.7-6.4)    | 4.7±0.40<br>(4.2-5.5)    | 4.8±0.44<br>(4.2-5.5)    | 5.5±0.41<br>(5.0-6.2)    |

| Trait | PSE<br><i>n</i> = 14     | MEN<br><i>n</i> = 10     | SAK<br><i>n</i> = 14     | USH<br><i>n</i> = 14     | UTS<br><i>n</i> = 15     | HID<br><i>n</i> = 7      |
|-------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| R3FL  | 3.7±0.61<br>(2.4-4.7)    | 3.7±0.47<br>(3.2-4.4)    | 4.1±0.40<br>(3.5-4.7)    | 3.4±0.67<br>(2.5-4.7)    | 3.7±0.67<br>(2.8-4.8)    | 4.0±0.15<br>(3.8-4.2)    |
| R3TL  | 8.3±1.21<br>(4.9-9.6)    | 8.0±1.21<br>(5.1-9.2)    | 8.0±0.60<br>(6.9-9.0)    | 7.8±0.64<br>(6.2-9.0)    | 8.3±0.75<br>(6.4-9.1)    | 7.7±0.30<br>(7.3-8.2)    |
| RIND  | 5.1±0.54<br>(4.2-6.1)    | 5.0±0.24<br>(4.7-5.4)    | 4.9±0.49<br>(3.7-5.5)    | 5.2±0.59<br>(4.7-7.0)    | 5.1±0.69<br>(3.9-6.1)    | 5.9±0.28<br>(5.4-6.2)    |
| RIOD  | 6.3±0.39<br>(5.8-6.9)    | 6.3±0.52<br>(5.5-7.0)    | 6.6±0.37<br>(5.9-7.1)    | 5.7±0.51<br>(4.5-6.4)    | 6.3±0.41<br>(5.6-7.0)    | 7.3±0.26<br>(7.0-7.8)    |
| RUEW  | 3.4±0.28<br>(3.1-4.0)    | 3.3±0.31<br>(3.0-4.1)    | 3.5±0.21<br>(3.2-3.8)    | 3.2±0.29<br>(2.8-3.7)    | 3.3±0.30<br>(2.9-3.7)    | 3.4±0.27<br>(3.1-3.9)    |
| RSL   | 6.5±0.42<br>(5.7-7.3)    | 6.5±0.49<br>(5.8-7.4)    | 6.4±0.32<br>(5.8-7.1)    | 6.3±0.42<br>(5.7-7.2)    | 6.6±0.54<br>(5.6-7.6)    | 7.0±0.43<br>(6.5-7.7)    |
| RUEL  | 4.7±0.33<br>(4.0-5.0)    | 4.6±0.34<br>(4.0-5.1)    | 4.7±0.24<br>(4.3-5.1)    | 4.4±0.21<br>(4.1-4.8)    | 4.6±0.29<br>(4.1-5.1)    | 4.7±0.14<br>(4.5-4.9)    |
| RLJL  | 14.4±0.87<br>(13.1-16.1) | 14.0±0.72<br>(12.8-15.4) | 14.8±0.79<br>(13.3-16.0) | 13.4±0.48<br>(12.5-14.2) | 14.2±0.71<br>(13.2-15.5) | 14.1±0.73<br>(13.3-15.1) |

Table 3. Significant values of the 21 morphological traits among six species

|                   | PSE vs.<br>MEN | PSE vs.<br>SAK | PSE vs. USH     | PSE vs. UTS | PSE vs. HID                 | MEN vs.<br>SAK | MEN vs.<br>USH             | MEN vs.<br>UTS  |
|-------------------|----------------|----------------|-----------------|-------------|-----------------------------|----------------|----------------------------|-----------------|
| SVL               | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RTRL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RAGD              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RHL               | NS             | NS             | <i>P</i> < 0.05 | NS          | NS                          | NS             | NS                         | NS              |
| RTAL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RMTAW             | NS             | NS             | NS              | NS          | NS                          | NS             | <i>P</i> < 0.01            | NS              |
| RMTAH             | NS             | NS             | NS              | NS          | <i>P</i> < 0.05             | NS             | <b><i>P</i> &lt; 0.001</b> | NS              |
| RVTL              | NS             | NS             | NS              | NS          | <i>P</i> < 0.01             | NS             | NS                         | NS              |
| RVTW              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RHW               | NS             | NS             | <i>P</i> < 0.05 | NS          | NS                          | NS             | <b><i>P</i> &lt; 0.001</b> | NS              |
| RFL               | NS             | NS             | NS              | NS          | <i>P</i> < 0.05             | NS             | NS                         | NS              |
| RHLL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | <i>P</i> < 0.05 |
| R2FL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| R3FL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| R3TL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RIND              | NS             | NS             | NS              | NS          | <i>P</i> < 0.05             | NS             | NS                         | NS              |
| RIOD              | NS             | NS             | <i>P</i> < 0.01 | NS          | <b><i>P</i> &lt; 0.0001</b> | NS             | <i>P</i> < 0.05            | NS              |
| RUEW              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RSL               | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RUEL              | NS             | NS             | NS              | NS          | NS                          | NS             | NS                         | NS              |
| RLJL              | NS             | NS             | <i>P</i> < 0.01 | NS          | NS                          | NS             | NS                         | NS              |
| <i>P</i> < 0.0001 | 0              | 0              | 0               | 0           | 1                           | 0              | 0                          | 0               |
| <i>P</i> < 0.001  | 0              | 0              | 0               | 0           | 0                           | 0              | 2                          | 0               |
| <i>P</i> < 0.01   | 0              | 0              | 2               | 0           | 1                           | 0              | 1                          | 0               |
| <i>P</i> < 0.05   | 0              | 0              | 2               | 0           | 3                           | 0              | 1                          | 1               |
| Total             | 0              | 0              | 4               | 0           | 5                           | 0              | 4                          | 1               |

Table 3. Significant values of the 21 morphological traits among six species ( continue )

|       | MEN vs. HID     | SAK vs. USH                | SAK vs. UTS     | SAK vs. HID     | USH vs. UTS     | USH vs. HID                 | UTS vs. HID                |
|-------|-----------------|----------------------------|-----------------|-----------------|-----------------|-----------------------------|----------------------------|
| SVL   | NS              | <i>P</i> < 0.05            | <i>P</i> < 0.05 | NS              | NS              | <b><i>P</i> &lt; 0.001</b>  | <i>P</i> < 0.01            |
| RTRL  | NS              | NS                         | NS              | NS              | NS              | NS                          | NS                         |
| RAGD  | NS              | NS                         | NS              | NS              | NS              | NS                          | NS                         |
| RHL   | NS              | <i>P</i> < 0.01            | NS              | NS              | NS              | NS                          | NS                         |
| RTAL  | NS              | NS                         | NS              | NS              | NS              | NS                          | NS                         |
| RMTAW | NS              | <b><i>P</i> &lt; 0.001</b> | NS              | NS              | NS              | <i>P</i> < 0.05             | NS                         |
| RMTAH | NS              | <b><i>P</i> &lt; 0.001</b> | NS              | NS              | <i>P</i> < 0.01 | <i>P</i> < 0.01             | NS                         |
| RVTL  | NS              | NS                         | NS              | NS              | NS              | <i>P</i> < 0.01             | <b><i>P</i> &lt; 0.001</b> |
| RVTW  | NS              | NS                         | NS              | NS              | NS              | NS                          | NS                         |
| RHW   | NS              | NS                         | NS              | <i>P</i> < 0.01 | NS              | <b><i>P</i> &lt; 0.001</b>  | <i>P</i> < 0.01            |
| RFL   | <i>P</i> < 0.01 | <i>P</i> < 0.01            | NS              | NS              | NS              | <b><i>P</i> &lt; 0.0001</b> | <i>P</i> < 0.01            |
| RHLL  | NS              | NS                         | NS              | NS              | NS              | NS                          | NS                         |
| R2FL  | NS              | <i>P</i> < 0.01            | <i>P</i> < 0.05 | NS              | NS              | <i>P</i> < 0.01             | <i>P</i> < 0.05            |

|              | MEN vs. HID | SAK vs. USH  | SAK vs. UTS | SAK vs. HID | USH vs. UTS | USH vs. HID  | UTS vs. HID  |
|--------------|-------------|--------------|-------------|-------------|-------------|--------------|--------------|
| R3FL         | NS          | $P < 0.05$   | NS          | NS          | NS          | NS           | NS           |
| R3TL         | NS          | NS           | NS          | NS          | NS          | NS           | NS           |
| RIND         | $P < 0.05$  | NS           | NS          | $P < 0.01$  | NS          | $P < 0.05$   | NS           |
| RIOD         | $P < 0.001$ | $P < 0.0001$ | NS          | $P < 0.01$  | $P < 0.01$  | $P < 0.0001$ | $P < 0.0001$ |
| RUEW         | NS          | $P < 0.05$   | NS          | NS          | NS          | NS           | NS           |
| RSL          | NS          | NS           | NS          | NS          | NS          | $P < 0.05$   | NS           |
| RUEL         | NS          | NS           | NS          | NS          | NS          | NS           | NS           |
| RLJL         | NS          | $P < 0.0001$ | NS          | NS          | $P < 0.05$  | NS           | NS           |
| $P < 0.0001$ | 0           | 2            | 0           | 0           | 0           | 2            | 1            |
| $P < 0.001$  | 1           | 2            | 0           | 0           | 0           | 2            | 1            |
| $P < 0.01$   | 1           | 3            | 0           | 3           | 2           | 3            | 3            |
| $P < 0.05$   | 1           | 3            | 2           | 0           | 1           | 3            | 1            |
| Total        | 3           | 10           | 2           | 3           | 3           | 10           | 6            |

Table 4. Characteristics among six species of *Hynobius*

| Character | Condition | PSE<br><i>n</i> = 14 | MEN<br><i>n</i> = 10 | SAK<br><i>n</i> = 14 | USH<br><i>n</i> = 14 | UTS<br><i>n</i> = 15 | HID<br><i>n</i> = 7 |
|-----------|-----------|----------------------|----------------------|----------------------|----------------------|----------------------|---------------------|
| MDL       | Absent    | 12 (85.7%)           | 7 (70%)              | 2 (14.3%)            | 7 (50%)              | 6 (40%)              | 7 (100%)            |
|           | Present   | 2 (14.3%)            | 3 (30%)              | 12 (85.7%)           | 7 (50%)              | 9 (60%)              | 0 (0%)              |
| DWSV      | Absent    | 3 (21.4%)            | 1 (10%)              | 0 (0%)               | 0 (0%)               | 3 (20%)              | 0 (0%)              |
|           | Present   | 11 (78.6%)           | 9 (90%)              | 14 (100%)            | 14 (100%)            | 12 (80%)             | 7 (100%)            |
| DWSL      | Absent    | 7 (50.0%)            | 1 (10%)              | 0 (0%)               | 0 (0%)               | 2 (13.3%)            | 0 (0%)              |
|           | Present   | 7 (50.0%)            | 9 (90%)              | 14 (100%)            | 14 (100%)            | 13 (86.7%)           | 7 (100%)            |
| DYBLDT    | Absent    | 4 (28.6%)            | 8 (80%)              | 5 (35.7%)            | 11 (78.6%)           | 1 (6.7%)             | 6 (85.7%)           |
|           | Present   | 10 (71.4%)           | 2 (20%)              | 9 (64.3%)            | 3 (21.4%)            | 14 (93.3%)           | 1 (14.3%)           |
| DYBLVT    | Absent    | 13 (92.9%)           | 10 (100%)            | 14 (100%)            | 14 (100%)            | 15 (100%)            | 7 (100%)            |
|           | Present   | 1 (7.1%)             | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)              |
| DGM       | Absent    | 13 (92.9%)           | 4 (40%)              | 14 (100%)            | 14 (100%)            | 12 (80.0%)           | 7 (100%)            |
|           | Present   | 1 (7.1%)             | 6 (60%)              | 0 (0%)               | 0 (0%)               | 3 (20%)              | 0 (0%)              |
| CGN       | 11        | 1 (7.1%)             | 1 (10%)              | 1 (7.1%)             | 1 (7.1%)             | 1 (6.7%)             | 0 (0%)              |
|           | 12        | 13 (92.9%)           | 8 (80%)              | 13 (92.9%)           | 11 (78.6%)           | 13 (86.7%)           | 6 (85.7%)           |
|           | 13        | 0 (0%)               | 1 (10%)              | 0 (0%)               | 2 (14.3%)            | 1 (6.7%)             | 1 (14.3%)           |
| CFBALN    | 2.0       | 1 (7.1%)             | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)              |
|           | 1.5       | 1 (7.1%)             | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)               | 1 (14.3%)           |
|           | 1.0       | 4 (28.6%)            | 0 (0%)               | 4 (28.6%)            | 1 (7.1%)             | 2 (13.3%)            | 3 (42.9%)           |
|           | 0.5       | 4 (28.6%)            | 0 (0%)               | 4 (28.6%)            | 2 (14.3%)            | 1 (6.7%)             | 3 (42.9%)           |
|           | 0.0       | 2 (14.3%)            | 0 (0%)               | 3 (21.4%)            | 5 (35.7%)            | 4 (26.7%)            | 0 (0%)              |
|           | -0.5      | 1 (7.1%)             | 6 (60%)              | 1 (7.1%)             | 5 (35.7%)            | 4 (26.7%)            | 0 (0%)              |
|           | -1.0      | 1 (7.1%)             | 3 (30%)              | 2 (14.3%)            | 1 (7.1%)             | 4 (26.7%)            | 0 (0%)              |
|           | -1.5      | 0 (0%)               | 1 (10%)              | 0 (0%)               | 0 (0%)               | 0 (0%)               | 0 (0%)              |

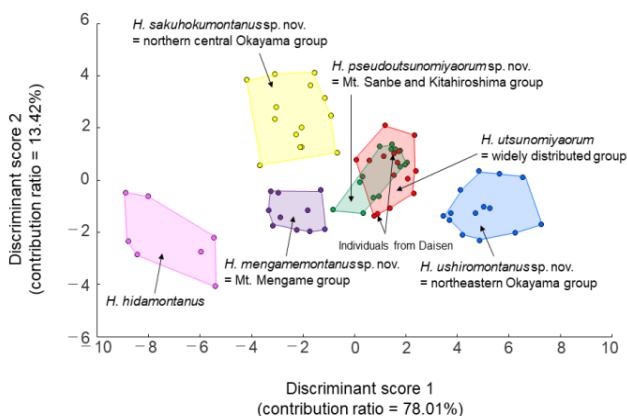


Figure 3. Result of canonical discriminant analysis in males

Canonical discriminant analysis results in males indicated distinctiveness among the six groups including *H. hidamontanus*, as evidenced by nonoverlapping distribution area scores. The only exception was observed between the Mt. Sanbe and Kitahiroshima and widely distributed groups (Figure 3: green, violet, yellow, blue,

red, and pink circles indicate the scores of the Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, northeastern Okayama, and widely distributed groups, and *H. hidamontanus*, respectively).

Morphological observations among the five groups of *H. utsunomiyaorum* and *H. hidamontanus* are shown in Table 4. PSE, MEN, SAK, USH, and UTS correspond to the Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, northeastern Okayama, and widely distributed groups, respectively, with HID representing *H. hidamontanus*. The Mt. Sanbe and Kitahiroshima group almost always had 12 CGN (13/14 = 92.9%), almost always had no DYBLVT (13/14 = 92.9%) and DGM (13/14 = 92.9%), usually had  $\geq 0.0$  CFBALN (12/14 = 85.8%), usually had no MDL (12/14 = 85.7%), and frequently had DWSV (11/14 = 78.6%) and DYBLDT (10/14 = 71.4%) (Table 4). The Mt. Mengame group always had no more than -0.5 CFBALN (10/10 = 100%), always had no DYBLVT (10/10 = 100%), almost always had DWSV (9/10 = 90%) and DWSL (9/10 = 90%), usually had 12 CGN (8/10 = 80%), usually had no DYBLDT (8/10 = 80%), and frequently had no MDL (7/10 = 70%) (Table 4). The northern central Okayama group always had DWSV (14/14

= 100%) and DWSL (14/14 = 100%), always had no DYBLVT (14/14 = 100%) and DGM (14/14 = 100%), almost always had 12 CGN (13/14 = 92.9%), usually had MDL (12/14 = 85.7%), and frequently had  $\geq 0.0$  CFBALN (11/14 = 78.6%) (Table 4). The northeastern Okayama group always had DWSV (14/14 = 100%) and DWSL (14/14 = 100%), always had no DYBLVT (14/14 = 100%) and DGM (14/14 = 100%), frequently had 12 CGN (11/14 = 78.6%) and  $\leq 0.0$  CFBALN (11/14 = 78.6%), and frequently had no DYBLDT (11/14 = 78.6%) (Table 4). The widely distributed group always had no DYBLVT (15/15 = 100%), almost always had DYBLDT (14/15 = 93.3%), usually had DWSV (12/15 = 80%), DWSL (13/15 = 86.7%), 12 CGN (13/15 = 86.7%), and  $\leq 0.0$  CFBALN (12/15 = 80.0%), and usually had no DGM (12/15 = 80%) (Table 4). *H. hidamontanus* always had DWSV (7/7 = 100%), DWSL (7/7 = 100%), and  $\geq 0.5$  CFBALN (7/7 = 100%), always had no MDL (7/7 = 100%), DYBLVT (7/7 = 100%), and DGM (7/7 = 100%), usually had 12 CGN (6/7 = 85.7%), and usually had no DYBLDT (6/7 = 85.7%) (Table 4).

Based on the three species concepts, the Mt. Sanbe and Kitahiroshima, Mt. Mengame, northern central Okayama, and northeastern Okayama groups of *H. utsunomiyaorum* were described as new species.

## 4. Systematics

### *Hynobius pseudoutsunomiyaorum* sp. nov.

ZooBank LSID: urn:lsid:zoobank.org:act:C5145D84-2E02-4C4A-899F-396BEE860049 (Figures 4-5)

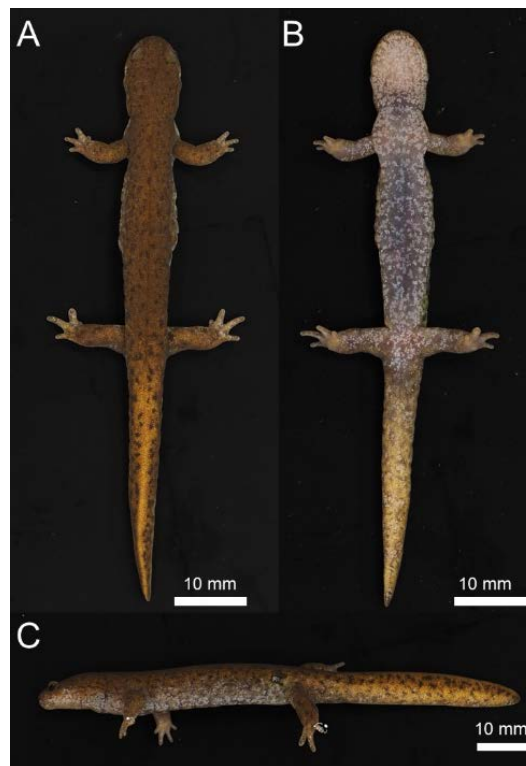
Synonymy: *Hynobius utsunomiyaorum* Matsui et al. (2019: clade B2b4, H9-H11).

**Holotype.** An adult male (specimen number: HMNH-AM-324) from Shinjo, Kitahiroshima Town, Hiroshima Prefecture, Chugoku, Japan (34° 46' N, 132° 29' E; elevation = 420 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Jun-ichi Naito on 17 March 2022. This specimen was deposited in the Shobara Municipal Hiwa Museum for Natural History: 1119-1, Hiwacho Hiwa, Shobara City, Hiroshima Prefecture, 727-0301, Japan.

**Paratypes.** An adult female (specimen number: YCM-RA598) from Yamaguchicho Yamaguchi, Oda City, Shimane Prefecture, Chugoku, Japan (35° 10' N, 132° 38' E; elevation = 380 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Takayuki Iwata on 26 February 2021. An adult male (specimen number: YCM-RA599) from the same locality, collected by Takayuki Iwata on 6 March 2020. The female and male specimens were deposited in the Yokosuka City Museum: 95 Fukadadai, Yokosuka City, Kanagawa Prefecture, 238-0016, Japan.

**Diagnosis.** A small species (mean snout-vent length = 52.6 mm in male) within the Japanese lentic *Hynobius*: mid-dorsal line usually absent; distinct white spots (or mottles) on venter frequently present; distinct yellowish-brown line on dorsal edge of the tail frequently present; distinct yellowish-brown line on ventral edge of the tail

almost always absent; distinct gular mottling almost always absent; almost always 12 costal grooves (rarely 11); number of costal folds between adpressed limbs usually  $\geq 0.0$ ; fifth toe of hindlimb always absent (or rudimentary); dorsal color very variable; U (or V) shaped vomerine teeth series; coil-shaped egg sacs.



**Figure 4.** Holotype of *Hynobius pseudoutsunomiyaorum* sp. nov.: (A) dorsal, (B) ventral, (C) lateral views.



**Figure 5.** *Hynobius pseudoutsunomiyaorum* sp. nov.: (A) adult, (B) larva, (C) type locality, (D) egg sacs

Description of holotype. A large individual: HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; unexpanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length  $II > III > IV > I$  in both sides; four toes on each hindlimb, order of length  $III > II > IV > I$  in both sides; U-shaped like vomerine teeth; skin smooth and shiny; distinct and scattered black spots on dorsum present; MDL absent; DWSV and DWSL present; DYBLDT present; DYBLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 53.7, TRL = 42.4, AGD = 29.5, HL = 12.1, TAL = 36.3, MTAW = 3.2, MTAH = 5.3, BTAW = 5.8, BTAH = 5.3, LVTL = 2.7, RVTL = 2.8, VTW = 2.8, HW = 8.8, LFL = 12.3, RFL = 12.6, LHLL = 14.9, RHLL = 14.5, L1FL = 1.1, L2FL = 2.6, L3FL = 2.1, L4FL = 1.2, R1FL = 0.9, R2FL = 2.1, R3FL = 2.0, R4FL = 1.1, L1TL = 0.7, L2TL = 2.6, L3TL = 3.8, L4TL = 1.5, R1TL = 1.2, R2TL = 2.9, R3TL = 3.6, R4TL = 1.8, IND = 2.7, IOD = 3.0, LUEW = 1.3, RUEW = 1.1, LSL = 3.7, RSL = 3.6, LUEL = 2.7, RUEL = 2.5, LLJL = 6.4, RLJL = 5.9, CGN = 12.

Comparisons. The new species does not differ statistically from *H. utsunomiyaorum* (Table 3), but frequently has  $\geq 0.5$  CFBALN (10/14 = 71.4%) (Table 4). The new species differs statistically from *H. hidamontanus* in the following length measurements: RMTAH, RVTL, RFL, RIND, and RIOD (Table 3). The lengths of these measurements, except for RVTL, are significantly shorter in *H. pseudoutsunomiyaorum* sp. nov. than in *H. hidamontanus* (Tables 2-3). DYBLDT is frequently present in *H. pseudoutsunomiyaorum* sp. nov. (10/14 = 71.4%) but usually absent in *H. hidamontanus* (6/7 = 85.7%) (Table 4). The distribution ranges of the two species are clearly separated (Figure 1). The new species is parapatrically distributed with *Hynobius iwami*, but it is distinguishable by the absence of a distinct yellow line on the ventral side of the tail [7]. In addition, it is parapatrically distributed with *Hynobius akiensis*, has a distinct fifth toe, and usually lacks a distinct brownish line on the dorsal side of the tail [25]. The new species and *Hynobius sematonotos* and *Hynobius kimurae* are distributed in proximity, but the former and latter species have distinct silver mottles and distinct yellow mottles, respectively, on the dorsal and lateral sides of the body [26,27]. *Hynobius sematonotos* and *H. kimurae* never have yellow or brown lines on the dorsal and ventral sides of the tail, although the latter species has longer vomerine teeth series [26,27].

Variation and coloration. Morphometric measurements and observations are presented in Tables 2 and 4, respectively. The new species rarely has DYBLVT (1/14 = 7.1%), DGM (1/14 = 7.1%), and 11 CGN (1/14 = 7.1%), rarely has MDL (2/14 = 14.3%) and  $\leq -0.5$  CFBALN (2/14 = 14.3%), rarely has no DWSV (3/14 = 21.4%) and DYBLDT (4/14 = 28.6%), and sometimes has DWSL (7/14 = 50.0%) (Table 4). Its dorsal coloration was variable and tends to fade to dark gray when preserved. Its ventral coloration was lighter than the dorsal coloration and tends to fade to a darker color when preserved.

Distribution. It is known from Oda City (including the former Oda City), Iinan (including the former Akagi and

Tonbara Towns) and Misato (including the former Ochi Town) Towns in Shimane Prefecture and Shobara City (including the former Takano Town) and Kitahiroshima Town (including the former Oasa Town) in Hiroshima Prefecture. The distribution record of Shobara City was only recorded by Matsui et al. [7] based on DNA data. The dominant vegetation type of the type locality is mixed forest of *Quercus crispula*, *Quercus serrata*, and *Cryptomeria japonica* (Figure 9).

Larvae and egg sacs. The breeding season of this species is February to April. Breeding locations are pools or ditches at forest edges. The larval and egg sac morphologies of this new species are similar to those of *H. utsunomiyaorum* [7]. Larvae usually have black dots on the body and tail (more densely on the tail), but body color is variable. Claws are absent from the fingers and toes of larvae. One pair of balancers are present during the early developmental stages of larvae. Larvae always metamorphose and land within the same year of hatching. Egg sacs are coiled without striations and attached to fallen branches or leaves.

Etymology. The specific name derives from the morphology of this species, which closely resembles that of *H. utsunomiyaorum*: *pseudo* (= false in Latin) + *utsunomiyaorum* (= specific name of Hiba salamander). The suggested Japanese common name is Nisehiba-sanshouo.

Remarks. The morphology of females is unknown; thus, further studies are needed to clarify the morphological characters of females.

### *Hynobius mengamemontanus* sp. nov.

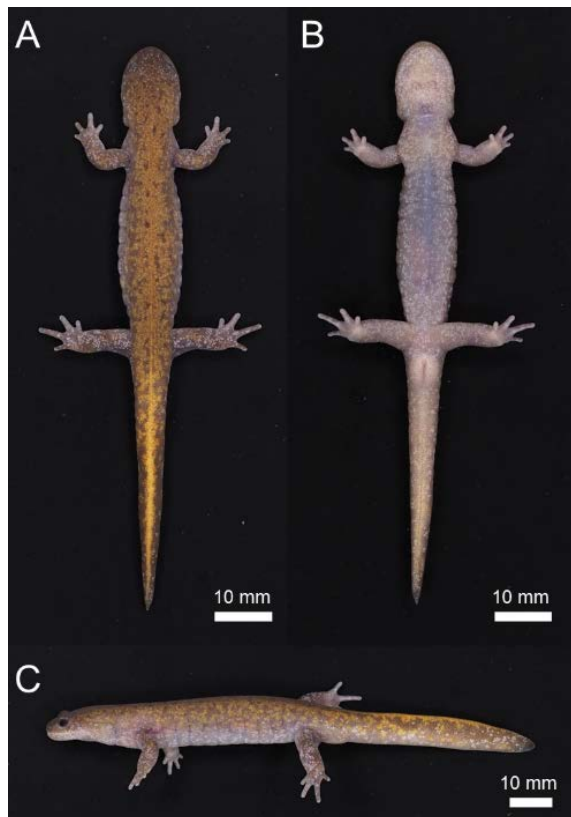
ZooBank LSID: urn:lsid:zoobank.org:act:D042FD71-C291-45F3-9179-E2181BA0D316 (Figures 6-7)

Synonymy: *Hynobius nebulosus* Okawa et al. (1990: Pops. 59-60).

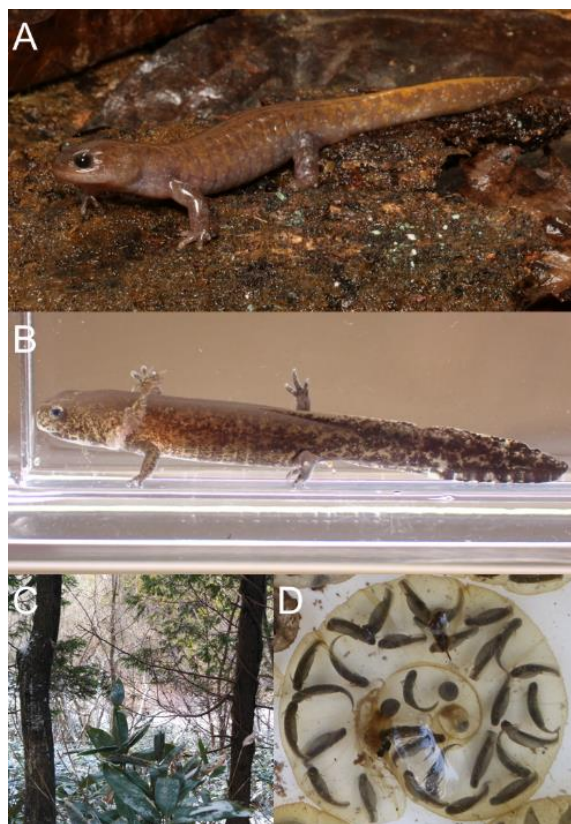
Holotype. An adult male (specimen number: HMNH-AM-325) from Sakugicho Okamibuchi, Miyoshi City, Hiroshima Prefecture, Chugoku, Japan (34° 56' N, 132° 41' E; elevation = 520 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Jun-ichi Naito on 14 December 2022. This specimen was deposited in the Shobara Municipal Hiwa Museum for Natural History.

Paratypes. An adult female (specimen number: YCM-RA600) and male (specimen number: YCM-RA601) from Kamiakana, Iinan Town, Shimane Prefecture, Chugoku, Japan (34° 57' N, 132° 43' E; elevation = 660 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Takayuki Iwata on 4 March 2021 and 21 March 2020, respectively. These specimens were deposited in the Yokosuka City Museum.

Diagnosis. A small species (mean snout-vent length = 52.4 mm in male) within the Japanese lentic *Hynobius*: mid-dorsal line frequently absent; distinct white spots (or mottles) on ventral and lateral sides of the body almost always present; distinct yellowish-brown line on dorsal edge of the tail usually absent; distinct yellowish-brown line on ventral edge of the tail always absent; usually 12 costal grooves (rarely 11 or 13); number of costal folds between adpressed limbs always  $\leq -0.5$ ; fifth toe of hindlimb always absent; dorsal color very variable; V (or U) shaped vomerine teeth series; coil-shaped egg sacs.



**Figure 6.** Holotype of *Hynobius mengamemontanus* sp. nov.: (A) dorsal, (B) ventral, (C) lateral views.



**Figure 7.** *Hynobius mengamemontanus* sp. nov.: (A) adult, (B) larva, (C) type locality, (D) egg sacs.

**Description of holotype.** A large individual: HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually

compressed toward the tip; slightly expanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length  $II > III > I > IV$  in left and  $II > I > III > IV$  in right; four toes on each hindlimb, order of length  $III > II > IV > I$  in both sides; V-shaped vomerine teeth; skin smooth and shiny; distinct black spots on dorsum present; MDL present on the latter half of dorsum; DWSV and DWSL present; DYBLDT present; DYBLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 55.4, TRL = 43.4, AGD = 29.4, HL = 12.1, TAL = 38.1, MTAW = 3.0, MTAH = 5.4, BTAW = 6.3, BTAH = 6.0, LVTL = 2.4, RVTL = 2.3, VTW = 2.8, HW = 10.0, LFL = 14.1, RFL = 12.8, LHLL = 16.6, RHLL = 16.9, L1FL = 1.0, L2FL = 3.0, L3FL = 1.5, L4FL = 0.5, R1FL = 1.5, R2FL = 3.0, R3FL = 1.3, R4FL = 0.7, L1TL = 1.6, L2TL = 3.1, L3TL = 4.2, L4TL = 2.0, R1TL = 1.5, R2TL = 3.2, R3TL = 4.2, R4TL = 1.9, IND = 2.7, IOD = 3.0, LUEW = 1.3, RUEW = 1.1, LSL = 4.2, RSL = 4.1, LUEL = 2.3, RUEL = 2.3, LLJL = 7.4, RLJL = 7.2, CGN = 12.

**Comparisons.** The new species statistically differs from *H. utsunomiyaorum* in RHLL (Table 3). The length of the measurement is significantly shorter in *H. mengamemontanus* sp. nov. than in *H. utsunomiyaorum* (Tables 2-3). DYBLDT usually absent in *H. mengamemontanus* sp. nov. (8/10 = 80.0%), but almost always present in *H. utsunomiyaorum* (14/15 = 93.3%) (Table 4). The new species statistically differs from *H. hidamontanus* in the following length measurements: RFL, RIND and RIOD (Table 3). The lengths of these measurements are significantly shorter in *H. mengamemontanus* sp. nov. than in *H. hidamontanus* (Tables 2-3). CFBALN always  $\leq -0.5$  in *H. mengamemontanus* sp. nov. (10/10 = 100%), but always  $\geq 0.5$  in *H. hidamontanus* (7/7 = 100%) (Table 4). Their distribution ranges are clearly separated (Figure 1). The new species statistically does not differ from *H. pseudotsunomiyaorum* sp. nov. (Table 3). DYBLDT usually absent in *H. mengamemontanus* sp. nov. (8/10 = 80%), but frequently present in *H. pseudotsunomiyaorum* sp. nov. (10/14 = 71.4%) (Table 4). CFBALN always  $\leq -0.5$  in *H. mengamemontanus* sp. nov. (10/10 = 100%), but usually  $\geq 0.0$  in *H. pseudotsunomiyaorum* sp. nov. (12/14 = 85.8%) (Table 4). The new species and *H. sematonotos* are geographically close, but the latter have distinct silver mottles on the dorsal and lateral sides of the body [27].

**Variation and coloration.** Morphometric measurements and observations are presented in Tables 2 and 4, respectively. The new species had rarely 11 (1/10 = 10%) or 13 (1/10 = 10%) CGN and DYBLDT (2/10 = 20%), occasionally had MDL (3/10 = 30%), occasionally had no DGM (4/10 = 40%), and rarely had no DWSV (1/10 = 10.0%) and DWSL (1/10 = 10.0%) (Table 4). The dorsal coloration was very variable, and it tended to fade to dark gray when preserved.

**Distribution.** It is known from Iinan Town (only former Akagi Town) in Shimane Prefecture and Miyoshi City (only former Sakugi and Funo Villages) in Hiroshima Prefecture. The distribution record of Funo Village was suggested by Okawa et al. [28]. The dominant vegetation

type in the type locality is Japanese cypress (*Chamaecyparis obtusa*).

**Larvae and egg sacs.** The breeding season of this species is March to April. Breeding places are pools or ditches at forest edges. Larvae always metamorphose and land within the same year of hatching. The larval and egg sacs morphologies of this new species are similar with *H. pseudotsunomiyaorum* sp. nov. and *H. utsunomiyaorum* [7].

**Etymology.** The specific epithet “*mengamemontanus*” derives from Mt. Mengame located in the northeastern part of Miyoshi City (Hiroshima Prefecture) and southern part of Iinan Town (Shimane Prefecture); the new species only occurs around this mountain. Although the mountain is called Mt. Mengame in Hiroshima Prefecture, it is called Mt. Megame in Shimane Prefecture. Many years ago, the mountain was called Mt. Megami, which means goddess in Japanese. Over time, “Megami” and “Mengami” were corrupted into “Megame” and “Mengame” in Shimane Prefecture and Hiroshima Prefecture, respectively. As the name of the mountain originates from Megami (= goddess), the suggested standard Japanese name is Megame-sanshouo.

**Remarks.** The new species likely forms a monophyletic group with *H. pseudotsunomiyaorum* sp. nov., but the monophyly was not supported statistically (Figure 2A). The morphology of females is unknown; thus, further studies are needed to clarify the morphological characters of females.

### *Hynobius sakuhekumontanus* sp. nov.

ZooBank LSID: urn:lsid:zoobank.org:act:2E4C0758-E62A-4A8E-A8D0-C2BBB66051A1 (Figures 8-9)

**Synonymy:** *Hynobius utsunomiyaorum* Matsui et al. (2019: clade B2b4, H6).

**Holotype.** An adult male (specimen number: TRPM-ARA-0000015) from Kamisaibara, Kagamino Town, Okayama Prefecture, Chugoku, Japan (35° 18' N, 133° 54' E; elevation = 780 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Takayuki Iwata on 3 May 2019. This specimen was deposited in the Tottori Prefectural Museum: 2-124, Higashimachi, Tottori City, Tottori Prefecture, 680-0011, Japan.

**Paratypes.** An adult female (specimen number: YCM-RA602) from the same locality of holotype, collected by Takayuki Iwata on 23 April 2021. An adult male (specimen number: YCM-RA603) from Hiruzenshitao, Maniwa City, Okayama Prefecture, Chugoku, Japan (35° 15' N, 133° 46' E; elevation = 490 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Masaru Yamada on 23 October 2021. These specimens were deposited in the Yokosuka City Museum.

**Diagnosis.** A small species (mean snout-vent length = 50.4 mm in male) within the Japanese lentic *Hynobius*: mid-dorsal line usually present; distinct white spots (or mottles) on ventral and lateral sides of the body always present; distinct yellowish-brown line on ventral edge of the tail always absent; distinct gular mottling always absent; almost always 12 costal grooves (rarely 11); number of costal folds between adpressed limbs frequently  $\geq 0.0$ ; fifth toe of hindlimb always absent (or

rudimentary); dorsal color very variable; V or U-shaped vomerine teeth series; coil-shaped egg sacs.



**Figure 8.** Holotype of *Hynobius sakuhekumontanus* sp. nov.: (A) dorsal, (B) ventral, (C) lateral views.



**Figure 9.** *Hynobius sakuhekumontanus* sp. nov.: (A) adult, (B) larva, (C) type locality, (D) egg sacs

Description of holotype. A large individual: HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; unexpanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length  $II > III > IV > I$  in both sides; four toes on each hindlimb, order of length  $III > II > IV > I$  in both sides; V-shaped vomerine teeth; skin smooth and shiny; distinct and scattered brownish-yellow spots on dorsum present; MDL present on the latter half of dorsum; DWSV and DWSL present; DYBLDT absent; DYBLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 56.3, TRL = 43.1, AGD = 29.6, HL = 13.3, TAL = 42.9, MTAW = 2.9, MTAH = 5.1, BTAW = 6.5, BTAH = 5.5, LVTL = 2.5, RVTL = 2.6, VTW = 2.7, HW = 9.5, LFL = 14.1, RFL = 14.7, LHLL = 17.7, RHLL = 16.9, L1FL = 0.8, L2FL = 2.6, L3FL = 2.4, L4FL = 1.0, R1FL = 0.9, R2FL = 2.7, R3FL = 2.4, R4FL = 1.1, L1TL = 1.3, L2TL = 2.9, L3TL = 4.1, L4TL = 2.7, R1TL = 1.4, R2TL = 3.4, R3TL = 4.0, R4TL = 1.9, IND = 2.7, IOD = 3.5, LUEW = 1.7, RUEW = 1.6, LSL = 3.6, RSL = 3.4, LUEL = 2.6, RUEL = 2.4, LLJL = 7.7, RLJL = 6.7, CGN = 12.

Comparisons. The new species statistically differs from *H. utsunomiyaorum* in the following length measurements: SVL and R2FL. The lengths of SVL and R2FL are significantly shorter and longer in *H. sakuhokumontanus* sp. nov. than in *H. utsunomiyaorum*, respectively (Tables 2-3). Their distribution ranges are slightly separated (Figure 1). The new species statistically differs from *H. hidamontanus* in the following length measurements: RHW, RIND, and RIOD (Table 3). The lengths of these measurements are significantly shorter in *H. sakuhokumontanus* sp. nov. than in *H. hidamontanus* (Tables 2-3). MDL usually present in *H. sakuhokumontanus* sp. nov. (12/14 = 85.7%), but always absent in *H. hidamontanus* (7/7 = 100%) (Table 4). Their distribution ranges are clearly separated (Figure 1). The new species statistically does not differ from *H. pseudotsunomiyaorum* sp. nov., but the new species usually has MDL (12/14 = 85.7%) towards *H. pseudotsunomiyaorum* sp. nov. usually has no MDL (12/14 = 85.7%) (Table 4). The distribution ranges of two species are clearly separated (Figure 1). Although the new species statistically does not differ from *H. mengamemontanus* sp. nov. (Table 3), CFBALN frequently  $\geq 0.0$  in *H. sakuhokumontanus* sp. nov. (11/14 = 78.6%) against always  $\leq -0.5$  (10/10 = 100%) in *H. mengamemontanus* sp. nov. (Table 4). Their distribution ranges are clearly separated (Figure 1). The new species and *H. sematonotos* and *H. kimurae* are geographically close, but the former has distinct silver mottles, latter has distinct yellow mottles on the dorsal and lateral sides of the body [26,27]. *Hynobius sematonotos* and *H. kimurae* never has yellow or brown lines on the dorsal and ventral sides of the tail, and latter has longer vomerine teeth series [26,27].

Variation and coloration. Morphometric measurements and observations are presented in Tables 2 and 4, respectively. The new species very rarely had 11 CGN (1/14 = 7.1%), rarely had  $\leq -0.5$  CFBALN (3/14 = 21.4%), rarely had no MDL (2/14 = 14.3%), occasionally had no DYBLDT (5/14 =

35.7%) (Table 4). The dorsal coloration was very variable, and it tended to fade to dark gray when preserved.

Distribution. This new species is endemic to Okayama Prefecture; it is known from Kagamino Town (only former Kamisaibara Village) and Maniwa City (only former Chuka Village). The dominant vegetation type in the type locality is *Quercus acutissima*.

Larvae and egg sacs. The breeding season of this species is usually April to May, but the breeding season of the population of former Chuka Village is December to January. Larvae usually metamorphose and land within the same year of hatching. However, there are confirmed instances of populations in which most individuals metamorphose and land in following year. Breeding places are pools or ditches at forest edges. The larval and egg sacs morphologies of this new species are similar with *H. pseudotsunomiyaorum* sp. nov. and *H. utsunomiyaorum* [7].

Etymology. The specific epithet "*sakuhokumontanus*" derives from the Sakuhoku Mountains located in the northern part of Okayama Prefecture where the new species occurs. The suggested standard Japanese name is Sakuhoku-sanshouo.

Remarks. The distribution ranges of this new species and *H. utsunomiyaorum* are separated, but re-examination of the record of *H. utsunomiyaorum* from Kagamino Town lacking detailed information about sampling points [7] is essential. The morphology of female is unknown, so further studies to clarify the morphological characters of females are needed.

### *Hynobius ushiromontanus* sp. nov.

ZooBank LSID: urn:lsid:zoobank.org:act:BC0BEB9-9E6E-467F-AE86-55256FD770DB (Figures 10-11)

Synonymy: *Hynobius utsunomiyaorum* Matsui et al. (2019: clade B2b4, H7-H8).

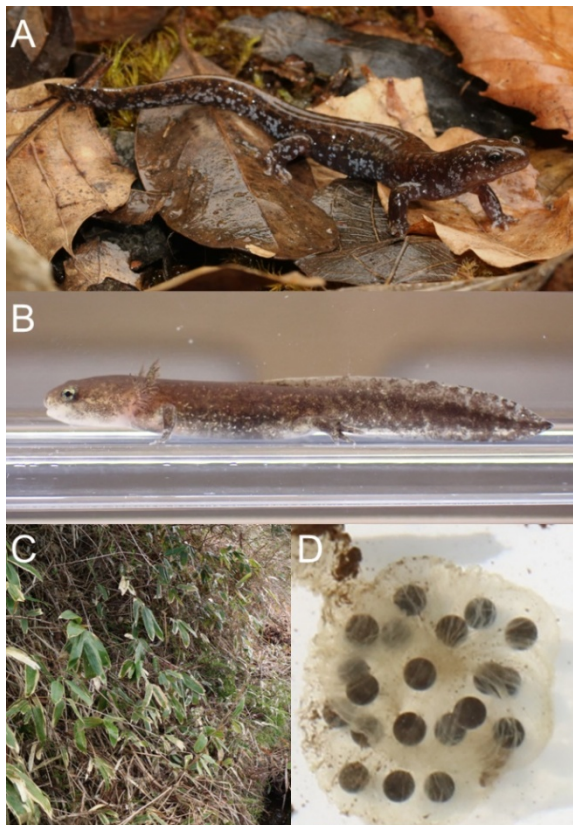
Holotype. An adult male (specimen number: TRPM-ARA-0000016) from Ogaya, Nishiawakura Village, Okayama Prefecture, Chugoku, Japan (35° 13' N, 134° 23' E; elevation = 1020 m above sea level [a.s.l.]; in all cases, datum = WGS84), collected by Takayuki Iwata on 2 May 2019. This specimen was deposited in the Tottori Prefectural Museum.

Paratypes. An adult female (specimen number: YCM-RA604) and male (specimen number: YCM-RA605) from the same locality of holotype, collected by Takayuki Iwata on 28 April 2021 and 2 May 2019. These specimens were deposited in the Yokosuka City Museum.

Diagnosis. A small species (mean snout-vent length = 55.5 mm in male) within the Japanese lentic *Hynobius*: distinct white spots (or mottles) on ventral and lateral sides of the body always present; distinct yellowish-brown line on dorsal edge of the tail frequently absent; distinct yellowish-brown line on ventral edge of the tail always absent; distinct gular mottling always absent; frequently 12 costal grooves (rarely 11 or 13); number of costal folds between adpressed limbs almost always  $\geq -0.5$ ; fifth toe of hindlimb always absent; dorsal color almost always brownish with black spots; V or U shaped vomerine teeth series; coil-shaped egg sacs.



**Figure 10.** Holotype of *Hynobius ushiromontanus* sp. nov.: (A) dorsal, (B) ventral, (C) lateral views.



**Figure 11.** *Hynobius ushiromontanus* sp. nov.: (A) adult, (B) larva, (C) type locality, (D) egg sacs.

Description of holotype. A moderately large individual: HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; unexpanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length  $II > III > I > IV$  in both sides; four toes on each hindlimb, order of length  $III > II > IV > I$  in both sides; V-shaped like vomerine teeth; skin smooth and shiny; distinct black spots on dorsum present; MDL present; DWSV and DWSL present; DYBLDT present; DYBLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 56.1, TRL = 43.5, AGD = 28.4, HL = 12.4, TAL = 40.9, MTAW = 3.2, MTAH = 5.2, BTAW = 6.0, BTAH = 4.9, LVTL = 2.6, RVTL = 2.5, VTW = 3.1, HW = 9.2, LFL = 13.6, RFL = 14.5, LHLL = 17.7, RHLL = 18.3, L1FL = 0.9, L2FL = 2.5, L3FL = 1.7, L4FL = 0.7, R1FL = 0.8, R2FL = 2.9, R3FL = 1.9, R4FL = 0.7, L1TL = 1.5, L2TL = 3.4, L3TL = 4.2, L4TL = 2.3, R1TL = 1.1, R2TL = 3.4, R3TL = 4.6, R4TL = 2.4, IND = 2.7, IOD = 3.6, LUEW = 1.0, RUEW = 0.9, LSL = 3.9, RSL = 3.5, LUEL = 2.5, RUEL = 2.3, LLJL = 7.4, RLJL = 7.2, CGN = 12.

Comparisons. The new species statistically differs from *H. utsunomiyaorum* in the following length measurements: RMTAH, RIOD, and RLJL (Table 3). Their lengths are significantly shorter in *H. ushiromontanus* sp. nov. than in *H. utsunomiyaorum* (Tables 2-3). DYBLDT frequently absent in *H. ushiromontanus* sp. nov. (11/14 = 78.6%), but almost always present in *H. utsunomiyaorum* (14/15 = 93.3%) (Table 4). Distribution ranges of two species are clearly separated (Figure 1). The new species statistically differs from *H. hidamontanus* in the following length measurements: SVL, RMTAW, RMTAH, RVTL, RHW, RFL, R2FL, RIND, RIOD, and RSL. The lengths of these measurements excepting for SVL and RVTL are significantly shorter in *H. ushiromontanus* sp. nov. than in *H. hidamontanus*. CFBALN frequently  $\leq 0.0$  in *H. ushiromontanus* sp. nov. (11/14 = 78.6%), but always  $\geq 0.5$  in *H. hidamontanus* (7/7 = 100%) (Table 4). Distribution ranges of two species are clearly separated (Figure 1). The new species statistically differs from *H. pseudotsunomiyaorum* sp. nov. in the following length measurements: RHL, RHW, RIOD, and RLJL (Table 3). Their lengths are significantly shorter in *H. ushiromontanus* sp. nov. than in *H. pseudotsunomiyaorum* sp. nov. (Tables 2-3). DYBLDT frequently absent in *H. ushiromontanus* sp. nov. (11/14 = 78.6%), but frequently present in *H. pseudotsunomiyaorum* sp. nov. (10/14 = 71.4%) (Table 4). CFBALN frequently  $\leq 0.0$  in *H. ushiromontanus* sp. nov. (11/14 = 78.6%), but frequently  $\geq 0.5$  in *H. pseudotsunomiyaorum* sp. nov. (10/14 = 71.4%) (Table 4). Distribution ranges of two species are clearly separated (Figure 1). The new species statistically differs from *H. mengamemontanus* sp. nov. in the following length measurements: RMTAW, RMTAH, HW, and RIOD (Table 3). Their lengths are significantly shorter in *H. ushiromontanus* sp. nov. than in *H. mengamemontanus* sp. nov. (Tables 2-3). Distribution ranges of then are clearly separated (Figure 1). The new species statistically differs from *H. sakuhokumontanus* sp. nov. in the following length measurements: SVL, RHL, RMTAW, RMTAH, RFL, R2FL, R3FL, RIOD, RUEW, and RLJL (Table 3). Their lengths excepting for SVL are significantly shorter

in *H. ushiromontanus* sp. nov. than in *H. sakuhokumontanus* sp. nov. (Tables 2-3). Distribution ranges of them are clearly separated (Figure 1). The new species and *H. sematonotos* and *H. kimurae* are geographically close, but the former has distinct silver mottles, latter has distinct yellow mottles on the dorsal and lateral sides of the body [26,27]. *Hynobius sematonotos* and *H. kimurae* never has yellow or brown lines on the dorsal and ventral sides of the tail, and latter has longer vomerine teeth series [26,27].

Variation and coloration. Morphometric measurements and observations are presented in Tables 2 and 4, respectively. The new species very rarely had 11 CGN (1/14 = 7.1%), rarely had 13 CGN (2/14 = 14.3%), rarely had DYBLDT (3/14 = 21.4%) and  $\geq 0.5$  CFBALN (3/14 = 21.4%), and sometimes had MDL (7/14 = 50%). The dorsal coloration is almost always brownish with black spots and it tended to fade to dark gray when preserved.

Distribution. It is known from Nishiawakura Village, Okayama Prefecture, and Shiso City (only former Chikusa Town), Hyogo Prefecture. The dominant vegetation type in the type locality is *Chamaecyparis obtusa*.

Larvae and egg sacs. The breeding season of this species is April to May. Breeding places are pools or ditches at forest edges. Larvae always metamorphose and land within the same year of hatching. The larval and egg sacs morphologies of this new species are similar with *H. pseudoutsunomiyaorum* sp. nov. and *H. utsunomiyaorum* [7].

Etymology. The specific epithet “*ushiromontanus*” derives from Mt. Ushiro located in the northeastern part of Okayama Prefecture and northwestern part of Hyogo Prefecture, and it occurs around the mountain. The suggested standard Japanese name is Ushiroyama-sanshou.

Remarks. The new species forms a monophyletic group with *H. sakuhokumontanus* sp. nov., although they are clearly distinct in terms of morphology (Table 3; Figure 3). The morphology of female is unknown, so further studies to clarify the morphological characters of females are needed.

### ***Hynobius utsunomiyaorum* Matsui et Okawa, 2019**

**Holotype.** An adult male (specimen number: KUHE 6491) from Rokunohara, Mt. Hiba, Saijocho Yuki, Shobara City, Hiroshima Prefecture (35°04'20"N, 133°04'18"E, alt. 850 m), collected by Masafumi Matsui on 3 May 1982 [7]. This specimen was deposited in the Graduate School of Human and Environmental Studies, Kyoto University: Yoshidahonmachi, Sakyo Ward, Kyoto City, Kyoto Prefecture, 606-8501, Japan [7].

**Diagnosis.** A small species (mean snout-vent length = 54.7 mm in male) within the Japanese lentic *Hynobius*: distinct white spots (or mottles) on ventral and lateral sides of the body usually present; distinct yellowish-brown line on dorsal edge of the tail almost always present; distinct yellowish-brown line on ventral edge of the tail always absent; distinct gular mottling usually absent; usually 12 costal grooves (very rarely 11 or 13); number of costal folds between adpressed limbs usually  $\leq 0.0$ ; fifth toe of hindlimb always absent (or rudimentary) except the populations from Daisen (Tottori Prefecture) and around areas; dorsal color very variable; U or V-shaped vomerine teeth series; coil-shaped egg sacs.

**Description of topotype.** An adult male (specimen number: YCM-RA606) from near the type locality (35°04'20"N, 133°04'18"E), collected by Takayuki Iwata on 3 April 2020 (Figure 12). This specimen was deposited in Yokosuka City Museum. A moderately large individual: HL larger than HW; TAL shorter than SVL; body almost cylindrical; rounded snout; gular fold present; tail gradually compressed toward the tip; unexpanded cloaca; webbing between digits absent; four fingers on each forelimb, order of length II > III > I > IV in both sides; four toes on each hindlimb, order of length III > II > IV > I in left side and III > IV > II > I in right side; U-shaped like vomerine teeth; skin smooth and shiny; distinct and scattered black spots on dorsum absent; distinct yellowish-brown mottling present on dorsum; MDL present on the latter half of dorsum; DWSV and DWSL present; DYBLDT present; DYBLVT absent; DGM absent. The holotype had the following measurements (in mm): SVL = 52.8, TRL = 41.1, AGD = 27.3, HL = 12.2, TAL = 39.7, MTAW = 2.7, MTAH = 4.9, BTAW = 5.6, BTAH = 4.8, LVTL = 2.2, RVTL = 2.0, VTW = 2.3, HW = 8.8, LFL = 16.1, RFL = 15.8, LHLL = 18.3, RHLL = 17.9, L1FL = 1.3, L2FL = 3.6, L3FL = 2.2, L4FL = 0.8, R1FL = 1.2, R2FL = 2.9, R3FL = 2.3, R4FL = 1.0, L1TL = 1.8, L2TL = 3.9, L3TL = 5.4, L4TL = 2.9, R1TL = 1.7, R2TL = 3.6, R3TL = 4.7, R4TL = 3.7, IND = 2.8, IOD = 3.0, LUEW = 1.0, RUEW = 1.1, LSL = 3.5, RSL = 3.9, LUEL = 2.2, RUEL = 2.0, LLJL = 6.4, RLJL = 6.2, CGN = 12.



**Figure 12.** Topotype of *Hynobius utsunomiyaorum*: (A) dorsal, (B) ventral, (C) lateral views

**Comparisons.** This species statistically differs from *H. hidamontanus* in the following length measurements: SVL, RVTL, RHW, RFL, R2FL, and RIOD (Table 3). The lengths of these measurements excepting for SVL and RVTL are significantly shorter in *H. utsunomiyaorum*

than in *H. hidamontanus* (Tables 2-3). DYBLDT almost always present in *H. utsunomiyaorum* (14/15 = 93.3%), but usually absent in *H. hidamontanus* (6/7 = 85.7%) (Table 4). Distribution ranges of then are clearly separated (Figure 1). This species is parapatrically distributed with *Hynobius kunibiki* and *Hynobius setoi*, but it is distinguishable in terms of the absence of distinct fifth toe and the distinct yellow line on ventral side of the tail [10]. *Hynobius utsunomiyaorum* is also distributed near the range of *H. akiensis*, but it has distinct fifth toe and usually lacks the distinct brownish line on dorsal side of the tail (at least in male) [7,25]. The new species and *H. sematonotos* and *H. kimurae* are geographically close, but the former has distinct silver mottles, latter has distinct yellow mottles on the dorsal and lateral sides of the body [26,27]. *Hynobius sematonotos* and *H. kimurae* never has yellow or brown lines on the dorsal and ventral sides of the tail, and latter has longer vomerine teeth series [26,27].

Variation and coloration. Morphometric measurements and observations are presented in Tables 2 and 4, respectively. The new species very rarely had 11 (1/15 = 6.7%) or 13 CGN (1/15 = 6.7%), rarely had DGM (3/15 = 20%) and  $\geq 0.5$  CFBALN (3/15 = 20%), very rarely had no DYBLDT (1/15 = 6.7%), rarely had no DWSL (2/15 = 13.3%) and DWSV (3/15 = 20%), and sometimes had no MDL (6/15 = 40.0%). The dorsal coloration is very variable, and it tended to fade to dark gray when preserved.

Distribution. Based on our field surveys and some articles, it is known from Tottori, Shimane, Okayama, and Hiroshima Prefectures: Kotoura (former Akasaki and/or Tohaku Towns), Daisen (only former Daisen Town), Hoki (former Kishimoto and Mizokuchi Towns), Nanbu (only former Aimi Town), Kofu, Hino, and Nichinan Towns, Tottori Prefecture, Matsue (only former Mihonoseki Town), Yasugi (only former Hakuta and Hirose Towns), and Unnan (only former Daito and Kisuki Towns and Yoshida Village) Cities, and Okuizumo (former Nita and Yokota Towns) and Iinan (only former Tonbara Town) Towns, Shimane Prefecture, Maniwa (only former Kawakami and Yatsuka Villages), Niimi (only former Tetta Town), and Takahashi (only former Bitchu Town) Cities, Okayama Prefecture, and Shobara City (former Shobara City, and Tojo, Saijo, Hiwa, Takano, Kuchiwa, and Soryo Towns) and Jinsekikogen Town (only former Jinseki, Yuki, and Sanwa Towns), Hiroshima Prefecture [28,29,30]. According to Matsui et al. [7], this species is also distributed in Kagamino Town (Pop. 71: Figure 1). Although the specific sampling points are unknown, the haplotype of this new species is not found in the area of former Kamisaibara Village based on our field surveys; therefore, its habitat area may be former Okutsu Town (or near the boundary of the Okutsu Town and Kamisaibara Village) located in the south side of Kamisaibara Village, i.e., an area that we were unable to survey in detail. This population shares a haplotype with Tetta Town (Pop. 64: Figures 1-2); thus, the new species may be distributed in the area located between these towns (i.e., former Niimi City and former Katsuyama and Kuse Towns of Maniwa City, located in BA2: Figure 1).

Larvae and egg sacs. The larval and egg sacs morphologies of this species are similar with *H. pseudotsunomiyaorum* sp. nov. Larvae usually metamorphose and land within the same year of hatching.

However, there are confirmed instances of populations in which most individuals metamorphose and land in following year. According to Matsui et al. [7], the breeding season of *H. utsunomiyaorum* is late March to late May, but our field surveys do not support this conclusion. The breeding season of *H. utsunomiyaorum* is revised as follows: from late December to late May. Breeding places are pools or ditches at forest edges.

Remarks. Based on the previous study [7], the lowest altitude record for known localities was 35 m. However, our field surveys contradict this finding, leading to a revision of the lowest altitude of the known locality to 5 m. The population of *H. utsunomiyaorum* from Daisen (Pops. 56, 69, and 72) differs from other *H. utsunomiyaorum* populations because it has a distinct fifth toe [7,31]. However, salamanders in that population are clearly polyphyletic (Figure 2), and overall morphometric differences are unclear (Figure 3).

## 5. Discussion

The first taxonomic investigation of *H. utsunomiyaorum* was performed by Matsui et al. [32]. According to this study, phylogenetic analyses using allozyme data did not support the monophyletic relationship of *H. utsunomiyaorum*, although sampling localities were limited [32]. The second taxonomic investigation of *H. utsunomiyaorum* was performed by Matsui et al. [7]. According to this study, *H. utsunomiyaorum* was a non-monophyletic group based on a mitochondrial phylogeny, but nuclear data indicated genetic similarity among the groups, although they were not monophyletic [7]. The third taxonomic investigation of *H. utsunomiyaorum* was conducted by Sugawara et al. [10]. In this study, the Izumo lineage of *H. utsunomiyaorum* suggested by Hayashi and Ooi [13] was described as *H. kunibiki*, and the polyphyly of *H. utsunomiyaorum* was partly resolved [10]. However, the validity of *H. hidamontanus* included in the clade of *H. utsunomiyaorum* based on the morphological, phylogenetic, and evolutionary species concepts was unconfirmed. Matsui et al. [7] conducted nuclear data analysis rather than not phylogenetic analyses, and the nuclear data of *H. hidamontanus* was not included, despite this species clearly being included in the clade of *H. utsunomiyaorum* [7,10]. Thus, the taxonomic validity of *H. hidamontanus* could not be assessed using the phylogenetic species concept. Based on the phylogenetic and evolutionary species concepts, *H. utsunomiyaorum* should be divided into five groups if *H. hidamontanus* is a valid species: *H. pseudotsunomiyaorum* sp. nov., *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov. + *H. ushiromontanus* sp. nov., *H. utsunomiyaorum*, and *H. hidamontanus*. From a morphological perspective, the morphological differences of *H. hidamontanus* were clear when each species was compared (Table 3; Figure 3). Although *H. pseudotsunomiyaorum* sp. nov. was not distinguishable from *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov., and *H. utsunomiyaorum* based on statistical tests (Table 3), the ranges of the discriminant scores of *H. mengamemontanus* sp. nov. and *H. sakuhokumontanus* sp. nov. did not overlap with the

range of *H. pseudotsunomiyaorum* sp. nov. (Figure 3). Additionally, *H. mengamemontanus* sp. nov. and *H. sakuhokumontanus* sp. nov. were distinguished from *H. pseudotsunomiyaorum* sp. nov. based on CFBALN (Table 4) and the frequency of the presence of MDL (as well as geographical isolation), respectively. Furthermore, the results of canonical discriminant analysis supported the morphological differentiation between *H. sakuhokumontanus* sp. nov. and *H. pseudotsunomiyaorum* sp. nov. (Figure 3). However, *H. pseudotsunomiyaorum* sp. nov. and *H. utsunomiyaorum* were similar with distribution areas that were not clearly separated (Figure 1). In terms of morphology, the discriminant scores of these two species overlapped based on morphometric data (Figure 3). Our analysis mainly depended on individuals from Pop. 4, which were slightly isolated from the main distribution area of *H. pseudotsunomiyaorum* sp. nov. If this population has coincidentally similar morphological characteristics with *H. utsunomiyaorum*, the morphology of *H. pseudotsunomiyaorum* sp. nov. may be similar to that of *H. utsunomiyaorum*. Thus, it is possible that morphometric differences between *H. pseudotsunomiyaorum* sp. nov. and *H. utsunomiyaorum* will be detected if individuals from other populations (except for Pops. 4 and 5) are examined. In Japanese *Hynobius*, two species may still be considered distinct species in some cases if the range of scores overlap [33]. CFBALN tended to be larger in *H. pseudotsunomiyaorum* sp. nov. than in *H. utsunomiyaorum* (Table 4). *Hynobius sakuhokumontanus* sp. nov. and *H. ushiromontanus* sp. nov. formed a monophyletic clade, consistent with the finding of Matsui et al. [7] (Figure 2), but they were clearly distinguishable based on the morphological species concept (Table 3; Figure 3). Furthermore, the distribution areas of the two species were clearly separated (Figure 1). Two groups may still be different species despite being monophyletic (e.g., *Hynobius sengokui* vs. *Hynobius tokyoensis* and *Hynobius tsurugiensis* vs. *Hynobius guttatus*) [33,34]. Moreover, *H. sakuhokumontanus* sp. nov. and *H. ushiromontanus* sp. nov. did not form a monophyletic clade based on the phylogeny using allozyme data [32]. Finally, we described the four new species and recognized two described species based on three species concepts. Results from comparisons using morphometric data did not reveal significant differences between *H. pseudotsunomiyaorum* sp. nov. vs. *H. mengamemontanus* sp. nov., *H. pseudotsunomiyaorum* sp. nov. vs. *H. sakuhokumontanus* sp. nov., *H. pseudotsunomiyaorum* sp. nov. vs. *H. utsunomiyaorum*, and *H. mengamemontanus* sp. nov. vs. *H. sakuhokumontanus* sp. nov. (Table 3), despite observed morphological differences being confirmed in these combinations (Table 4). To ascertain whether these combinations indeed exhibit significant differences in morphometric data, a more extensive study involving additional individuals and localities of the four species (*H. pseudotsunomiyaorum* sp. nov., *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov., and *H. utsunomiyaorum*) is warranted. This study was conducted without female individuals. To accumulate the taxonomic perspectives of the six species using this study, it is necessary to perform morphological comparisons among them using female individuals in a future study.

The population of *H. utsunomiyaorum* from Daisen (Pops. 56, 69, and 72) exhibits morphological differences in some characters [31] and generally has a fifth toe [7]; therefore, it was previously suggested that the Daisen populations may be a new species [31]. The present study included the Daisen population, but it was polyphyletic (Figure 2B) without conspicuous morphological differences (Figure 3). Thus, the populations around Daisen are likely a morphological variation of *H. utsunomiyaorum* characterized by the presence of a fifth toe.

The phylogenetic problem of *H. utsunomiyaorum* was resolved in the present study, but the phylogenetic problem of *H. hidamontanus* remains. *Hynobius tenuis*, described by Nambu [35], is a junior synonym of *H. hidamontanus* [36]. This synonymization was assigned based on the morphological and phylogenetic species concepts using skull characters and allozyme data [36]. Skull morphology is not absolutely needed for species description according to Matsui et al. [7]; therefore, it is possible the two species are different species based on the morphological species concept if other as yet undetected morphological differences exist between *H. hidamontanus* and *H. tenuis*. However, phylogenetic analyses using allozyme data were performed previously using UPGMA and neighbor-joining methods [36], and these methods are insufficient for constructing a robust molecular phylogenetic tree to assess the phylogenetic and evolutionary species concepts [37]. Moreover, the taxonomic perspectives of *Hynobius nebulosus* based on the allozyme data of Matsui et al. [32] were contradicted by Matsui et al. [7]; thus, the synonymization of *H. tenuis* using allozyme data lacks credibility. Furthermore, Matsui et al. [36] constructed a phylogeny without conducting genetic surveys across the distribution range of *H. tenuis* (i.e., using only two sampling points). Thus, detailed morphological comparisons and molecular phylogenetic analyses are needed to confirm the species validity of *H. tenuis* across the entire distribution range of *H. hidamontanus*.

The distribution area of *H. utsunomiyaorum* has been changed following the present description, i.e., it is not distributed in Hyogo Prefecture (Table 1; Figure 1). Although the distribution range of *H. utsunomiyaorum* decreased, *H. utsunomiyaorum* still has a wider distribution range than those of the other four new species (Figure 1). The estimated distribution ranges of *H. pseudotsunomiyaorum* sp. nov., *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov., and *H. ushiromontanus* sp. nov. are approximately less than 120, 10, 30, and 20 km<sup>2</sup>, respectively. In Japanese *Hynobius*, the species with the narrowest distribution range is *H. tosashimizuensis*, i.e., around 0.35 km<sup>2</sup> [4]. The scale of the distribution ranges of *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov., and *H. ushiromontanus* sp. nov. are larger than that of *H. tosashimizuensis*, but the narrow distribution ranges may be comparable with that of *Hynobius amakusaensis* [5]. The distribution range of *H. pseudotsunomiyaorum* sp. nov. is wider than that of the other three new species, but it is narrower than those of *H. hidamontanus* and *Hynobius abei* [38], which are well-protected by local governments in law (e.g., Internationally Rare Species of Wild Fauna/Flora).

The rank of *H. utsunomiyaorum* in the Japanese Red Data List is “vulnerable” (VU) [39]. However, the conservation status of the four new species should be reappraised. To conserve these species, it is necessary to conduct ecological studies on them (e.g., migratory distance, life-span, or survival rate in habitats) or the environment of their habitats (e.g., soil temperature, water temperature, or dissolved oxygen concentration of their breeding ponds). Therefore, ecological studies of these species should be conducted at the earliest opportunity to avoid their extinction.

## 6. Conclusions

The clade of Hiba salamander, *H. utsunomiyaorum* (sensu lato), including *H. hidamontanus* was divided into six species, and the four new species that were described based on the three species concepts used in this study are as follows: *H. pseudotsunomiyaorum* sp. nov., *H. mengamemontanus* sp. nov., *H. sakuhokumontanus* sp. nov., and *H. ushiromontanus* sp. nov.. The distribution areas between *H. pseudotsunomiyaorum* sp. nov. vs. *H. mengamemontanus* sp. nov., *H. pseudotsunomiyaorum* sp. nov. vs. *H. utsunomiyaorum*, and *H. sakuhokumontanus* sp. nov. vs. *H. utsunomiyaorum* are peripatric or parapatric based on our field surveys; therefore, introgression between them may have occurred currently or in the recent past. Moreover, there is a possibility of genetic pollution due to human activities. Therefore, studies are required on conservation genetics using microsatellite or single nucleotide polymorphism markers to elucidate the detailed genetic structure of the population.

## ACKNOWLEDGEMENTS

We are grateful to Toshiaki Iwata and Kazue Iwata for help with field surveys and sampling; Shoko Kumasaki, Nobue Akai, Masayuki Kitagawa, and Masashi Yamamoto for providing habitat information of the species used in this study; and Shingo Nakamura and Masaki Shindoh of Shobara Municipal Hiwa Museum for Natural History, Kei Ichisawa of Tottori Prefectural Museum, and Kiyoshi Hagiwara of Yokosuka City Museum for supporting the registration of the type specimens used in this study; the governments of Nagano Prefecture (permission number: 3 HOKUACHISO DAI 14 GO) and Hakuba Village (permission number: 2 HAKUKYOSYO DAI 55 GO) for giving the collecting permission of *H. hidamontanus*. We would like to thank Enago for the English review of this manuscript. This study was funded by JSPS KAKENHI, grant number JP18K02583.

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