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# Molecular Phylogenetic Position of *Abbottina springeri* (Cypriniformes; Cyprinidae) Based on Nucleotide Sequences of *RAG1* Gene

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**ABSTRACT** Partial nucleotide sequences of nuclear protein-coding recombination activating gene 1 (*RAG1*) gene of two *Abbottina* and five *Microphysogobio* species residing in Korea were analyzed to elucidate the molecular phylogenetic position of *A. springeri* Bánárescu and Nalbant. In *RAG1* tree *A. rivularis* was clearly separated from the monophyletic lineage composed of *A. springeri, Biwia zezera* and *Microphysogobio* species. Within this lineage *B. zezera* showed sister-group relationship to the monophyletic group composed of *A. springeri* and five *Microphysogobio* species. Thus, our phylogenetic tree revealed the polyphyletic nature of two *Abbottina* species from Korea, which result is well congruent with the previous phyletic assumption based on osteological features. The current classification of *Abbottina* and *Microphysogobio* based on morphological criteria, such as the presence or absence of papillae on lips and size of swim bladder with or without encapsulation, does not reflect their true evolutionary history.

**Key words :** *Abbottina rivularis*, *Abbottina springeri*, *Microphysogobio*, phylogeny, *RAG1*

## INTRODUCTION

The genus *Abbottina* is characterized by smooth and non papillose lips with the lower one with a single pad, and the large and free swim bladder (Bánárescu, 1992). Six nominal species (*A. binhi*, *A. lalinensis*, *A. liaoningensis*, *A. obtusirostris*, *A. rivularis* and *A. springeri*) are reported to inhabit throughout East Asia (FishBase: http://www.fishbase.org/). Among them *A. springeri* Bánárescu and Nalbant endemic to Korea was first reported by Bánárescu and Nalbant (1973), and Kim (1984) redescribed its morphological characters and geographical distribution. However its phylogenetic position is open to debate. Although Bánárescu and Nalbant (1973) included this species into the genus *Abbottina* because of the apparent lack of papillae on lips, it was treated as a species of the genus *Biwia* based on cephalic lateralis and osteology (Hosoya, 1986; Kawase and Hosoya, 2010). On the contrary, its osteological features are highly similar to *Microphysogobio* species, and Kang (1991) suggested transferring it to the genus *Microphysogobio*.

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Nuclear protein-coding recombination activating genes (*RAG1* and *RAG2*) are critically involved in genomic rearrangement events known as V(D)J recombination, which assembles a diverse repertoire of T-cell receptors (TCRs) and immunoglobulin (Ig) genes during lymphocyte developments (Schatz *et al.*, 1989; Fugmann *et al.*, 2000; De and Rodgers, 2004). The two genes, immediately adjacent in vertebrate genomes, synergistically activate V(D)J recombination (Oettinger *et al.*, 1990). Because of the critical roles the active core region for the endonuclease activity of *RAG1* gene is well conserved through the vertebrate evolution. The gene is present as a single-copy gene and encodes relatively large polypeptides without many indels in five fish genomes available in Ensembl (http://www.ensembl.org/). Moreover recent efforts developed pairs of conserved primers to successfully amplify a fragment of the third exon (exon 3) of *RAG1* gene (López *et al.*, 2004; Chen *et al.*, 2007; this study). All of these molecular properties are important requirements as a desirable phylogenetic marker for tracing natural evolutionary history (Li *et al.*, 2007; Chen *et al.*, 2008), which were applied to phylogenetic studies across diverse vertebrate taxa (e.g., Groth and Barrowclough, 1999; Venkatesh *et al.*, 2001; Vieites *et al.*, 2007). This

**Table 1.** Information on *Abbottina* and *Microphysogobio* species analyzed in this study

| Species | Voucher no.* | Sampling date | Sampling site | GenBank acc. no. |
|---|---|---|---|---|
| *Abbottina rivularis* | SUC-1876 | 24 Jul. 2010 | Masan-myeon, Seocheon-gun, Chungcheongnam-do | HQ699725 |
| *Abbottina springeri* | SUC-0828 | 14 Apr. 2010 | Gwangsi-myeon, Yesan-gun, Chungcheongnam-do | HQ699726 |
| *Microphysogobio jeoni* | SUC-1900 | 27 Jul. 2010 | Jangpyeong-myeon, Cheongyang-gun, Chungcheongnam-do | HQ699727 |
| *Microphysogobio koreensis* | SUC-1245 | 11 Jun. 2010 | Jigok-myeon, Hamyang-gun, Gyeongsangnam-do | HQ699728 |
| *Microphysogobio longidorsalis* | SUC-0806 | 01 May 2010 | Munmak-eup, Wonju-si, Gangwon-do | HQ699729 |
| *Microphysogobio rapidus* | SUC-1468 | 25 Jun. 2010 | Danseong-myeon, Sancheong-gun, Gyeongsangnam-do | HQ699730 |
| *Microphysogobio yaluensis* | SUC-0795 | 01 May 2010 | Munmak-eup, Wonju-si, Gangwon-do | HQ699731 |

*SUC means Soonchunhyang University Collection at the Department of Marine Biotechnology of Soonchunhyang University (Asan, South Korea)

marker was also widely used to resolve phylogenetic relationships among teleost fishes (López *et al.*, 2004; Sullivan *et al.*, 2006; Chen *et al.*, 2007; Šlechtová *et al.*, 2007; Conway *et al.*, 2008; Mayden *et al.*, 2008).

In spite of great controversy on taxonomy, there are no nucleotide sequence data for *A. springeri* and its related species (e.g., *A. rivularis* and five *Microphysogobio* species) residing in Korea except a few report (Im *et al.*, 2004). In this study we analyzed their nucleotide sequences of *RAG1* to elucidate the molecular phylogenetic position of *A. springeri*.

## MATERIALS AND METHODS

### 1. Specimen and genomic DNA extraction

Fish specimens of two *Abbottina* and five *Microphysogobio* species were captured with a spoon net (mesh size: 4 × 4 mm) from rivers or streams of South Korea. The voucher specimens were deposited in the collection of the Department of Marine Biotechnology of Soonchunhyang University (SUC; Asan, South Korea). Their detailed sampling information was shown in Table 1.

A piece of a pectoral or anal fin was excised from each specimen to extract genomic DNA. It was incubated with TNES-Urea buffer (10 mM Tris-HCl, pH 8.0; 125 mM NaCl; 10 mM EDTA, pH 8.0; 1% SDS; 8 M urea; Asahida *et al.*, 1996) containing 100 μg of proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C overnight, followed by separation of the aqueous phase with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) solution and ethanol precipitation according to Sambrook and Russell (2001). The extracted genomic DNA was finally resspended in 1 × TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Its quantity and quality were checked using a spectrophotometer, NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and by electrophoresis in a 0.7% agarose gel after staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

### 2. PCR amplification and sequencing

In order to amplify the exon 3 fragment of *RAG1* gene PCR was carried out in a 20-μL reaction volume using *AccuPower*® PCR Premix (Bioneer, Daejeon, Korea), including 50 ng of genomic DNA and 5 pmol of forward and reverse primers [RAG1-1495f3 (5′-CAGTAYCAY AAGATGTACCG-3′) and RAG1-3067r (5′-TTGTGAG CYTCCATRAACTT3′)], newly designed in this study. PCR was run with the following thermal cycling profile in a DNA Engine DYAD™ Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA): an initial denaturation at 94˚C for 3 min, 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and elongation at 72˚C for 1 min. The reaction was completed with a final elongation at 72˚C for 7 min. The PCR product was purified with the *AccuPrep*® PCR Purification Kit (Bioneer). After cycle sequencing with the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA, USA), the product was directly sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems Inc.) with the two PCR primers by a commercial company, Macrogen Inc. (Seoul, Korea). Electropherograms were assembled into a contig in Sequencher™ (Gene Codes Corp., Ann Arbor, MI, USA) and corrected manually. The sequences analyzed in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers HQ699725-HQ699731.

### 3. Phylogenetic analysis

Nucleotide sequences of *Abbottina* and *Microphysogobio* species analyzed in this study and those of gobionine species retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were aligned with ClustalW in BioEdit 7.0.5 (Hall, 1999) and refined manually. For reconstructing phylogenetic tree *Rhodeus ocellatus kurumeus* CTOL00544 (GenBank accession number: EU711142) and *Acheilognathus tabira tabira* CTOL01541 (EU409617) were used as outgroups. The nucleotide sequence matrix for *RAG1* consisted of 1,488 bp.

Neighbor-joining (NJ) analysis was carried out in PAUP* 4.0b10 (Swofford, 2002). NJ tree was reconstructed with the Kimura 2-parameter model. Robustness of tree topologies was evaluated by bootstrap analysis with 1,000 pseudoreplicates (Felsenstein, 1985).

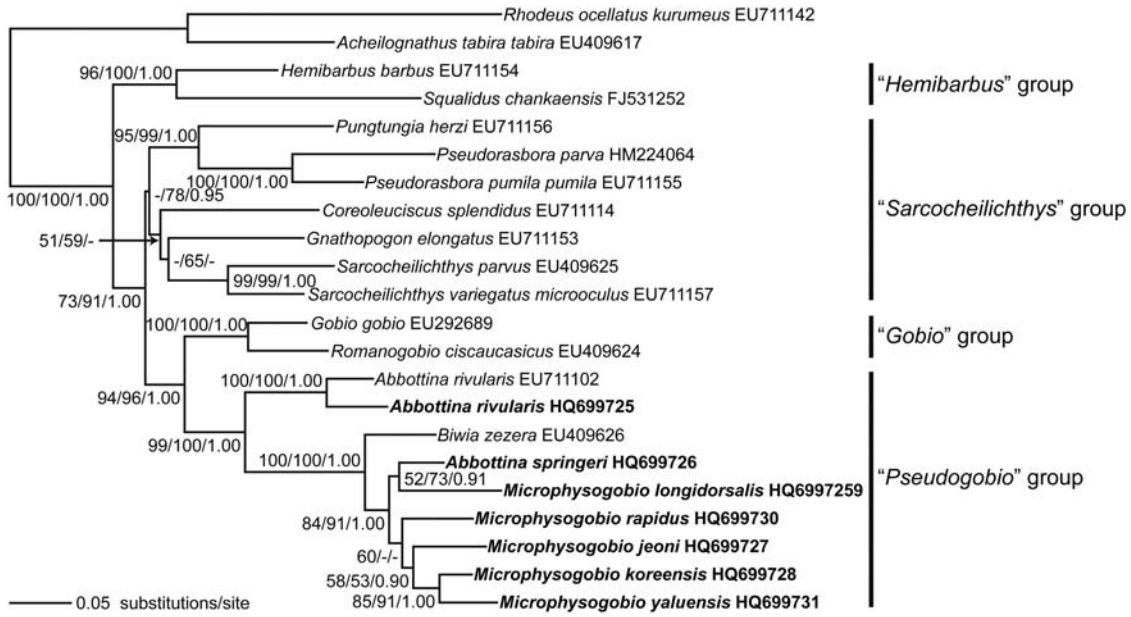


**Fig. 1.** Neighbor-joining tree of gobionine species inferred from nuclear protein-coding recombination activating gene 1 (*RAG1*) sequences. Bootstrap values above 50% for neighbor-joining and maximum likelihood analyses and posterior probabilities above 0.90 of Bayesian inference analysis were shown at each branch node, respectively. Species analyzed in this study are bold faced. Phylogenetic groupings according to Yang *et al.* (2006).

Maximum likelihood (ML) analysis was performed in RAxML 7.0.4 (Stamatakis, 2006; Stamatakis *et al.*, 2008). We executed the RAxML search for the best-scoring ML tree in one single program run (the "-f a" option) instead of the default maximum parsimony starting tree. The best-scoring ML tree of a thorough ML analysis was determined under the GTRGAMMAI model in 200 inferences. Statistical support was evaluated with 1,000 non-parametric bootstrap inferences.

Bayesian inference (BI) analysis was carried out in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with the GTR+I+Γ model, as in ML analysis. Two independent Metropolis-coupled Markov chain Monte Carlo (MCMCMC) runs were performed with four simultaneous chains (three heated and one cold) and random starting trees for 5,000,000 generations, sampling parameters and topologies every 100 generations. Burn-in was determined by checking the convergence of likelihood values across MCMCMC. A total of 500 out of 50,001 resulting trees were discarded as "burn-in". The last trees after convergence were used to construct a 50% majority-rule consensus tree and summarize posterior probability support for each node in PAUP*.

## RESULTS AND DISCUSSION

In the molecular phylogenetic tree based on *RAG1* sequences gobionine species were recovered as the monophyly with highest statistical supports in all tree-making algorithms (i.e., NJ, ML and BI methods), and ramified into four groups (Fig. 1), of which topologies are well congruent with the previous studies based on *COB* sequences (Yang *et al.*, 2006; Liu *et al.*, 2010). *Abbottina*, *Biwia* and *Microphysogobio* species belonging to "*Pseudogobio*" group (Yang *et al.*, 2006; Liu *et al.*, 2010) formed the strongly supported monophyletic group, with the sisterhood relationship to *Gobio* and *Romanogobio* species belonging to "*Gobio*" group. Within the "*Pseudogobio*" group two *A. rivularis* specimens consistently clustered together and placed at the basal position to the monophyletic lineage composed of *A. springeri*, *B. zezera* and *Microphysogobio* species with highest statistical supports. Within this lineage *B. zezera* showed the sisterhood relationship to the monophyletic group composed of *A. springeri* and five *Microphysogobio* species, of which phylogenetic affiliation was supported by 84 and 91% bootstrap values in NJ and ML trees, respectively, and 1.00 posterior probability in BI tree. Among them, *A. springeri* emerged together with *Microphysogobio* species with the closest phylogenetic affiliation to *M. longidorsalis*. Their phylogenetic affiliation was relatively weakly supported by 52 and 73% bootstrap values in NJ and ML trees, respectively, and 0.91 posterior probability in BI tree. *Microphysogobio jeoni*, *M. koreensis* and *M. yaluensis* were also recovered as the monophyly in all tree-making algorithms with more or less weak statistical supports, with the consistent clustering of the

latter two species. Meanwhile *M. rapidus* showed the unclear phylogenetic position among *A. springeri* and other *Microphysogobio* species, with the possible relationship to the clade of *M. jeoni*, *M. koreensis* and *M. yaluensis*, which was supported only in NJ tree by 60% bootstrap value.

Bánárescu and Nalbant (1973) first reported *A. springeri* as a novel species owing to the lack of papillae on lips, but mentioned its close resemblance to *B. zezera* rather than *A. rivularis* in the presence of a strong notch on the inside of gill-opening and in the shape of pharyngeal bones and teeth. Later Hosoya (1986) transferred this species to the genus *Biwia* (also see Kawase and Hosoya, 2010) based on cephalic line system and osteology, and Kang (1991) suggested transferring it to the genus *Microphysogobio* based on osteological features. Our phylogenetic tree inferred from *RAG1* sequences clearly revealed the polyphyletic nature of two *Abbottina* species from Korea; two specimens of *A. rivularis* placed at the basal position whereas *A. springeri* emerged among five *Microphysogobio* within *Pseudogobio* group molecular phylogenetic affiliation of *A. springeri* is well congruent with Kang's (1991) phyletic assumption. Thus the taxonomic position of *A. springeri* should be reappraised. However *A. springeri* has not been captured from its type locality or the connected main stem river, the Nakdong River basin, after its first report (Bánárescu and Nalbant, 1973; Kim, 1984), and it was impossible to include such in our phylogenetic analyses prevents us from reaching the final conclusion on the taxonomic position of *A. springeri*.

Meanwhile the sisterhood relationship of *B. zezera* to *Microphysogobio* species (and *A. springeri*) recovered in our phylogenetic tree is congruent with Hosoya's (1986) and Kang's (1991) phyletic assumptions. *Microphysogobio* species are well characterized by the combination of peculiar disposition of papillae on lips, deep suborbitals and reduced swim bladder with anterior chamber encapsulated, whereas *Abbottina* species by smooth and non papillose lips with the lower one with a single pad, and the large and free swim bladder (Bánárescu, 1992). Thus the current classification system of *Abbottina* and *Microphysogobio* based on morphological criteria such as the presence or absence of papillae on lips and size of swim bladder with or without encapsulation appears not to reflect their natural evolutionary history. However our phylogenetic tree included only Korean *Abbottina* and *Microphysogobio* species, although much diverse species were reported from the Mainland China, and did only one *Biwia* species despite the recent report of a novel species, *B. yodoensis* (Kawase and Hosoya, 2010). To reach the concrete final conclusion on intergeneric relationships among *Abbottina*, *Biwia* and *Microphysogobio* taxon samplings have to be extended for those species in the future study.

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# *RAG1* 유전자의 염기서열에 기초한 왜매치 *Abbottina springeri* (잉어목, 잉어과)의 분자계통학적 위치

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**요 약 :** 왜매치 *Abbottina springeri* Bánárescu and Nalbant의 분자계통학적 위치를 밝히기 위해 한국에 서식하는 버들매치속 2종과 모래주사속 5종의 핵 유전자인 recombination activating gene 1 (*RAG1*)의 염기서열을 분석하였다. *RAG1* 유전자 염기서열 정보에 기초한 계통수에서 배들매치 *A. rivularis*는 단계통군을 형성하는 왜매치, *Biwia zezera* 및 모래주사속 종들과 분리되었다. 이 계통 내에서 *B. zezera*는 왜매치와 모래주사속 5종을 구성된 단계통 그룹과 자매계통 관계를 보였다. 분자계통수 상에서 버들매치속 2종은 다계통군으로 나타났고, 이러한 결과는 골격 특징들에 근거한 이들의 계통적 관계를 밝힌 선행연구와 잘 일치하였다. 따라서 입의 피질돌기 유무와 부레의 골낭 유무와 크기 등과 같은 형태적 특징들에 근거한 버들배치속과 모래주사속의 현분류체계는 진화역사를 잘 반영하지 못하는 것으로 여겨진다.

**찾아보기 낱말 :** 버들매치, 왜매치, 모래주사속, 분자계통, *RAG1* 유전자