

# Genetic Divergence and Identification of Two Controversial Lanternfishes (Actinopterygii: Myctophidae: *Diaphus*) Based on Mitochondrial Cytochrome *b* Sequences and PCR-RFLP Analysis

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To resolve the controversial taxonomic status of two closely-related lanternfishes, *Diaphus perspicillatus* (Ogilby, 1898) and *D. gigas* Gilbert, 1913, mitochondrial cytochrome *b* (cyt *b*) gene sequences of representative individuals were obtained, and specifically-diagnostic restriction sites investigated. In the 5'-half nucleotide sequences of the cyt *b* gene (594 bp), a relatively high level of nucleotide substitution between the species (8.2–8.7%), together with very low intraspecific variation (<0.86%), was observed, which clearly indicates the two morphs to be specifically distinct. Previous hypotheses regarding gigantism of *D. gigas* and suspicions regarding the synonymy of the two species are rejected. The nucleotide sequences and practical restriction enzyme assay indicate that any one of four restriction endonucleases (*Fok* I, *Hae* III, *Nla* IV, and *Rsa* I) can unambiguously discriminate between the two species. Based on specimens identified by molecular analysis, eye diameter and gill raker count were found to be good diagnostic morphological characters.

**Key Words:** lanternfish, *Diaphus*, mtDNA, cyt *b*, PCR-RFLP, genetic divergence, identification.

## Introduction

The taxonomic status of two lanternfishes, *Diaphus perspicillatus* (Ogilby, 1898) and *D. gigas* Gilbert, 1913 (Myctophidae), has been controversial, although Kawaguchi and Shimizu (1978) recognized them as closely-related but distinct species that exhibit ecological and morphological differences. The former is distributed widely in tropical to temperate regions of all oceans (Kawaguchi and Shimizu 1978; Paxton 1979; Hulley 1981) whereas the latter is limited to temperate and subarctic regions of the North Pacific (Wisner 1976; Kawaguchi and Shimizu 1978; Kubodera and Furuhashi 1987; Kubota *et al.* 1989). Off the Pacific coast of the

Kanto region, Japan, the two species occur allopatrically in transitional waters (Fujii 1984). Adults of *D. gigas* have a larger body size (102.8–159.7 mm in standard length [SL]) than those of *D. perspicillatus* (24.5–66.8 mm SL) (data from Kubota *et al.* 1991), and the two species have also been distinguished by the positions of the third supra-anal (SAO<sub>3</sub>) and posterolateral (Pol) luminescent organs relative to the lateral line (Fig. 1), eye diameter relative to standard length, and numbers of pelvic rays, gill rakers (upper and lower limb, and total), and pyloric caeca (Kawaguchi and Shimizu 1978; Kawaguchi 1986; Kubota *et al.* 1991).

These differences notwithstanding, Kawaguchi and Shimizu (1978) also referred to Nafpaktitis' hypothesis (as pers. comm.) that *D. gigas* may represent oversized senile expatriates of warm-water *D. perspicillatus*, because of the apparent lack of juveniles as well as young and gravid females, and the adjacent and continuous distribution of the two species. Paxton (1979) also suspected that *D. gigas* might be a junior synonym of *D. perspicillatus*. Although Kawaguchi and Shimizu (1978) proposed the positions of two luminescent organs (SAO<sub>3</sub> and Pol) to be discriminating features, one of the present authors (KU) has become aware through a

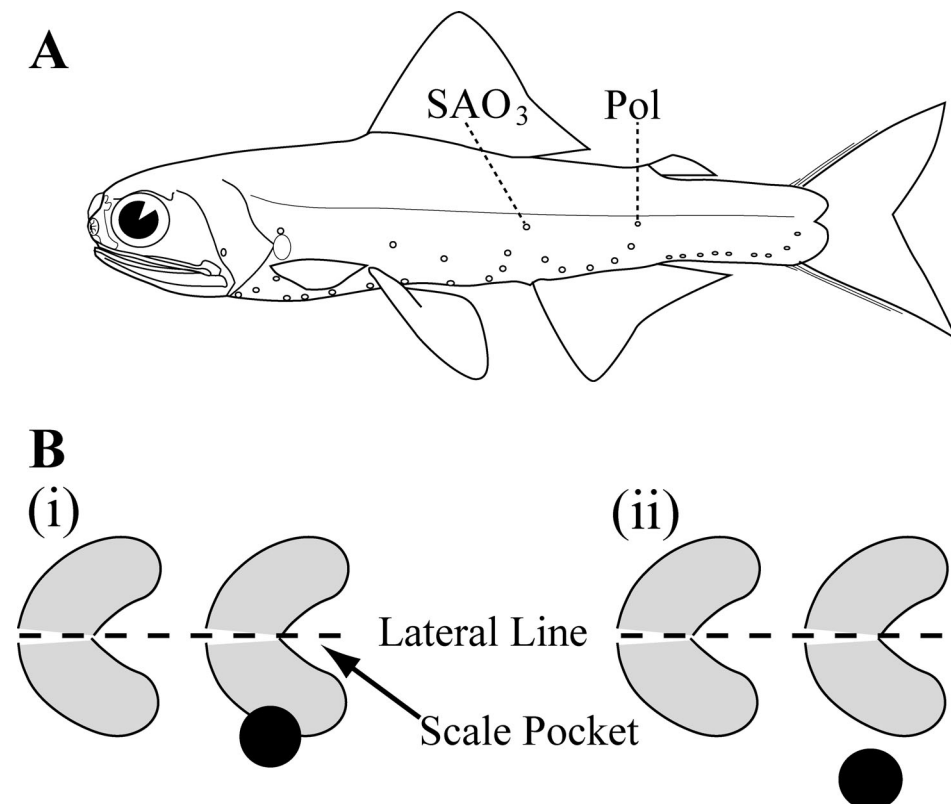


Fig. 1. General pattern of luminescent organs. A, *Diaphus gigas*, third supra-anal and posterolateral luminescent organs indicated as SAO<sub>3</sub> and Pol, respectively; B, relative positions of SAO<sub>3</sub> and Pol against lateral line in *D. perspicillatus* (i) and *D. gigas* (ii). Solid circle indicates luminescent organ.

morphological survey of the Myctophidae that these positions vary frequently among individuals and even from side to side in a single specimen. These characters are thus invalid to separate the two species. In sum, taxonomic agreement regarding these two nominal species has not been accomplished to date, the paucity of information on morphological development, maturity cycles, and species migrations, as well as the scarcity of young or juvenile individuals of *D. gigas* (see Wisner 1976), also being factors.

Mitochondrial DNA (mtDNA) analysis has become a conventional tool for assessing genetic divergence and has been used to identify fish species at all life stages (Chow *et al.* 1993; Aoyama *et al.* 2001; Sezaki *et al.* 2001; Takeyama *et al.* 2001; McDowell *et al.* 2002; Chow *et al.* 2003). The results of the present study, a nucleotide sequence analysis of the 5'-half of the cytochrome *b* (cyt *b*) genes of *D. perspicillatus* and *D. gigas*, appear to have resolved the above taxonomic problem. Moreover, diagnostic and conventional restriction fragment length polymorphism (RFLP) analyses are presented for discriminating between the two species.

## Materials and Methods

### Fish samples and DNA preparation

Following Kawaguchi and Shimizu (1978) and Kubota *et al.* (1991), specimens examined here were preliminarily identified as *D. perspicillatus*, having the eye diameter greater than 9.5% SL and the total number of gill rakers greater than 27, or *D. gigas*, the eye diameter less than 9.0% SL and the gill rakers fewer than 27. Seventeen specimens were used for mtDNA analysis, including four individuals of uncertain identity which were temporarily identified as *D. gigas* based on the above characters but rather small (44.0–48.0 mm SL) and with slight damage to the cephalic region. All of the specimens are deposited in the Research Faculty of Fisheries Sciences, Hokkaido University (HUMZ) (Table 1).

Fish samples were collected by RV *Shunyo-Maru* (National Research Institute

Table 1. fSpecimens examined in this study.

Species	Date of capture	Locality	N	SL (mm)	HUMZ No.
<i>Diaphus perspicillatus</i>	31 May 2003	23°56'N, 124°29'E, south of Yaeyama Islands	5	45.8–67.8	191827–191831
	1 June 2003	23°58'N, 124°29'E, south of Yaeyama Islands	2 <sup>a</sup>	62.3–65.7	191832, 191833
<i>D. gigas</i>	14 Oct. 2003	38°23'N, 141°58'E, off Kinka-zan	3 <sup>a</sup>	91.2–94.6	191834–191836
	14 Oct. 2003	38°23'N, 142°03'E, off Kinka-zan	2	85.4–108.2	191837, 191838
	May–June 1997	36–39°N, 150–153°E, east of Tohoku	4 <sup>b</sup>	44.0–48.0	191839–191842
	May–June 1997	36–39°N, 150–153°E, east of Tohoku	1	79.4	191843

N: sample size.

<sup>a</sup>Two specimens each used as representatives for DNA sequencing.

<sup>b</sup>Cephalic parts damaged during capture.

of Far Seas Fisheries) operating around the Yaeyama Islands, Okinawa, and RV *Wakataka-Maru* (Tohoku National Fisheries Research Institute) and RV *Tanshu-Maru* (Hyogo Prefectural Kasumi Senior High School) operating off the Tohoku region. The fish were frozen onboard immediately after capture. Subsequently transferred to the laboratory, they were identified and crude DNA samples were extracted from muscle tissues preserved in 70% ethanol, using the GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences).

### PCR amplification and nucleotide sequencing for representative specimens

From two representative individuals of each species, DNA fragments containing the entire *cyt b* gene were amplified for nucleotide sequencing *via* the polymerase chain reaction (PCR) using the primer pair CytB1F and CB6ThrH-15930, which amplified the target fragments best of the primer pairs considered in preliminary experiments. The forward primer CytB1F (5'-ATGGCAAGCCTCCGAAA-AAC-3'; Chow *et al.* 2003) is located at the 5'-end of the *cyt b* gene, and the reverse primer CB6ThrH-15930 (5'-CTCCAGTCTTCGGCTTACAAG-3'; slightly modified from Palumbi *et al.* 1991) lies within the tRNA<sup>Thr</sup> gene. PCR amplification followed Chow *et al.* (2003). Amplified products were purified using the PCR Product Pre-Sequencing Kit (USB), nucleotide sequences being generated on an automated sequencer (ABI Prism 310) using the ABI BigDye Ready Reaction Kit (Applied Biosystems), following standard cycle sequencing protocols. All sequences were obtained using only the forward PCR primer. They were aligned by comparison with the complete mitochondrial genome of *D. splendidus* (Brauer, 1904) (Miya *et al.* 2001). One of two alignments each from *D. perspicillatus* (HUMZ191832) and *D. gigas* (HUMZ191835) is available (accession numbers AB189992 and AB189993, respectively) from DDBJ/EMBL/GENBANK.

### PCR-RFLP analysis

The nucleotide sequences obtained from the representative specimens were imported into NEBcutter V2.0 (NEW ENGLAND BioLabs, <http://tools.neb.com/NEBcutter2/index.php>) for investigating all of the restriction sites. Of those sites, the ones expected to show distinctive restriction profiles between *D. perspicillatus* and *D. gigas* were selected and subjected to PCR-RFLP analysis. Simpler restriction profiles were obtained from the remaining specimens, shorter fragments than for the initial sequencing being amplified using a different reverse primer, CytB614R (5'-GAACCTGTTTCGTGAAGGAA-3', this study), that was designed to anneal to the middle of the *cyt b* gene. Amplification conditions for the shorter fragments followed those used for the longer fragments. The 2  $\mu$ l of amplified products were directly digested by endonucleases for three hours or longer, elec-

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Fig. 2. Representative nucleotide sequences of 5'-half region of the cytochrome *b* (*cyt b*) gene for *Diaphus perspicillatus* (HUMZ191832) and *D. gigas* (HUMZ191835). Nucleotide position numbers determined in this study and those of the corresponding region of the complete sequence for *D. splendidus* are shown in brackets and parentheses, respectively, along the right side margin. Restriction sites expected to effectively discriminate between *D. perspicillatus* and *D. gigas* are represented by turnover characters. The primer (CytB614R) sequence used for PCR-RFLP analysis, aligned complementarily, is also included.





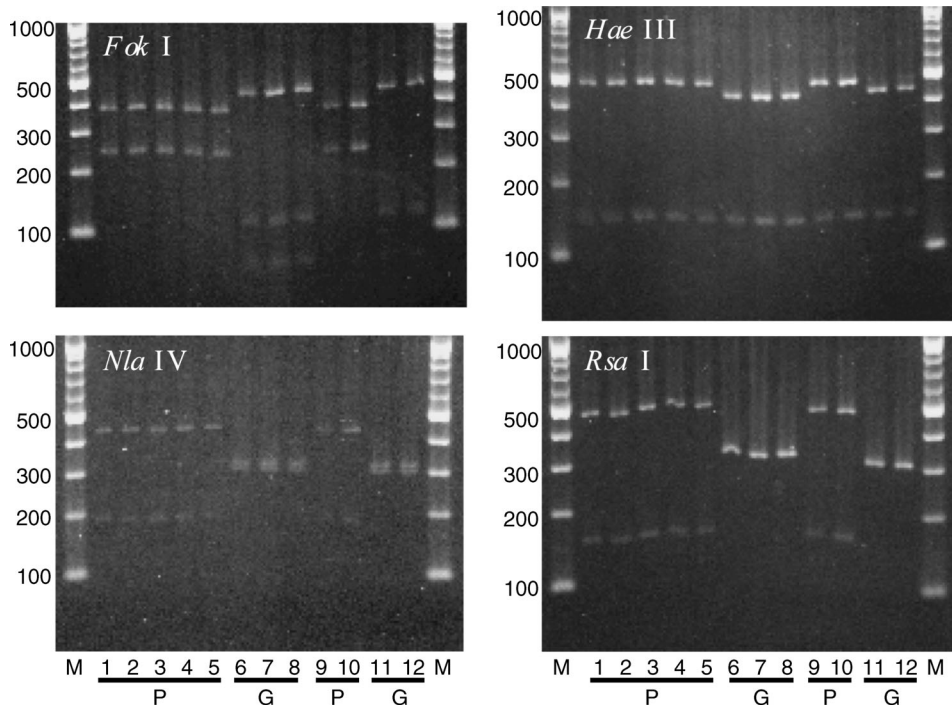


Fig. 3. PCR-RFLP pattern profiles of 5'-half fragment of the *cyt b* gene digested by four restriction endonucleases. Both sides of each photograph include molecular markers (100 bp DNA ladder, Amersham Biosciences), and the size is given on the left. "P" and "G" indicate samples from specimens morphologically identified as *Diaphus perspicillatus* and *D. gigas*, respectively. Lanes 9 to 12 are from sequenced specimens of each species.

### PCR-RFLP profiles

The shorter *cyt b* fragments (614 bp length including primer regions; Fig. 2) were used to investigate restriction fragment length polymorphism (RFLP) in seven and six specimens of *D. perspicillatus* and *D. gigas*, respectively, including the representative specimens previously sequenced. All four of the selected restriction endonucleases unambiguously discriminated between the two species, with no variation within species (Fig. 3). All restriction profiles were also congruent with those expected from the representative nucleotide sequences. This PCR-RFLP procedure was also applied to the four smaller individuals preliminarily identified as *D. gigas*. All of them were found to share identical restriction profiles with *D. gigas* adults (data not shown).

### Morphological comparisons

Since all of the present specimens could be certainly identified by sequencing and PCR-RFLP analyses, the purportedly diagnostic morphological taxonomic characters were compared and assessed (Table 3). Eye diameter of *D. perspicillatus* and *D. gigas* ranged between 9.8–11.0% SL and 7.8–8.8% SL, respectively, in agree-



Table 3. Comparisons of morphological characters of *Diaphus perspicillatus* and *D. gigas*.

Species	Eye diameter in % SL (mean±SD)		Gill rakers in total number (mean±SD)	
	This study	Kubota <i>et al.</i> (1991)	This study	Kubota <i>et al.</i> (1991)
<i>D. perspicillatus</i>	9.8–11.0 (10.4±0.403)	9.9–12.1	27–29 (28.0±0.667)	26–30 (28.0±0.845)
<i>D. gigas</i>	7.8–8.8 (8.17±0.282)	7.3–9.4	24–26 (25.1±0.594)	24–28 (25.7±0.854)

ment with Kubota *et al.* (1991). Ranges of total gill raker counts of both species (27–29 and 24–26, respectively) did not overlap in this study, contrary to the observations of Kubota *et al.* (1991).

### Discussion

Intra- and inter-specific divergences in the *cyt b* gene of marine fishes have been estimated as 0.8–4.3% and 2.8–17.6%, respectively, for three species of *Scomber* (Scoles *et al.* 1998), 0.2–0.6% and 4.4–8.2% for three species of *Aulostomus* (Bowen *et al.* 2001), and 0% and 11.8% (maximum divergence) for 23 species of the rockfish genus *Sebastes* (Kai *et al.* 2003). The genetic divergence observed in this study between the two lanternfishes (8.2–8.7%) clearly exceeded the intraspecific level, being comparable to those between congeners; therefore, we conclude that *D. perspicillatus* and *D. gigas* are specifically distinct and reject earlier hypotheses with respect to gigantism of *D. gigas*.

The present PCR-RFLP analysis clearly indicates that the four smaller specimens (44.0–48.0 mm SL) are young individuals of *D. gigas*, possibly the first reliable records of such, although Wisner (1976) recognized and described a few small specimens (45–57 mm SL) from the north-central Pacific as *D. gigas* based on their agreement in body proportions and numbers of gill rakers with Gilbert's (1913) description. Wisner (1976) stated that *D. gigas*, in general, had a more slender body, shorter and shallower head, and relatively smaller eye than *D. perspicillatus*, comparing measurements of his samples to those of the holotype of *D. gigas* (140 mm SL) and samples of *D. perspicillatus* (21–56 mm SL), including data from Nafpaktitis (1968).

Kawaguchi and Shimizu (1978) considered the taxonomy of western Pacific and eastern Indian Ocean *Diaphus* fishes, including six specimens of *D. perspicillatus* (37.5–56.5 mm SL) and eight of *D. gigas* (102.5–136.0 mm SL). Morphological differences between *D. perspicillatus* and *D. gigas* were presented as follows; (1) SAO<sub>3</sub> and Pol luminescent organs touching the lateral line in the former, but about one (organ) diameter below the lateral line in the latter (see Fig. 1), and (2) eye diameter greater than 9.5% SL in the former, less than 9.0% SL in the latter. Kubota *et al.* (1991) confirmed these differences using 121 specimens of *D. perspicillatus* (24.5–66.8 mm SL) and 157 of *D. gigas* (102.8–159.7 mm SL), in addition to listing several meristic differences between the species, *viz.* mean numbers of pectoral fin rays 10.992 and 11.295; gill rakers 9.975 and 8.395 (upper), 17.042 and 16.287 (lower), and 28.008 and 25.688 (total); and pyloric caeca 8.829 and 6.643, in *D. perspicillatus*



and *D. gigas*, respectively. However, neither paper included a comparison of similarly-sized specimens because of the lack of young *D. gigas*.

Of these morphological differences, some are problematic. The ranges in pectoral fin ray numbers largely overlap between the two species (Kubota *et al.* 1991), and the positions of SAO<sub>3</sub> and Pol vary between individuals or from side to side on single specimens, as mentioned above. Although the number of pyloric caeca is distinct between *D. perspicillatus* and *D. gigas* (Kubota *et al.* 1991), abdominal incision and counting is impractical for species identification. For these reasons, preliminary morphological identifications in this study were made using two external characters (relative eye diameter and gill raker count). The identifications so made were entirely consistent with the results from the PCR-RFLP analysis, indicating that these two features are reliable characters for distinguishing *D. perspicillatus* and *D. gigas* specimens of over *ca* 45 mm SL (Table 3). Relative eye diameter was found to be distinct between the two species, in agreement with Kubota *et al.* (1991), and we judge this to be the most reliable diagnostic character. Ranges of gill raker counts were discrete in this study, being adequate for discriminating between the two species, but this may be due to the smaller sample size than in Kubota *et al.* (1991).

It is likely that smaller individuals of *D. gigas* have been misidentified as *D. perspicillatus* in the past. Although the present results confirmed the morphological distinctiveness of the two lanternfish species over 45 mm SL, much smaller (< 45 mm; SL) specimens, particularly of *D. gigas*, should be examined at least by PCR-RFLP analysis to ascertain whether or not eye diameter and gill raker count are stable throughout all life stages of the species. The procedure for mtDNA analysis and diagnostic RFLP profiles proposed in this study may also aid the identification of specimens that are badly damaged during capture and should be a valuable identification tool for future studies of early life histories of the two species.

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