Zoological Studies **43**(4): 803-812 (2004)

Zoological Studies

Variable Numbers of Tandem Repeats (VNTRs), Heteroplasmy, and Sequence Variation of the Mitochondrial Control Region in the Three-spot Dascyllus, *Dascyllus trimaculatus* (Perciformes: Pomacentridae)

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(Accepted July 22, 2004)

Chaolun Allen Chen, Maria Carmen Anonuevo Ablan, John Williams McManus, Johann Diepernk Bell, Vo Si Tuan, Annadel Sarmiento Cabanban, Kwang-Tsao Shao (2004) Variable numbers of tandem repeats (VNTRs), heteroplasmy, and sequence variation of the mitochondrial control region in the three-spot Dascyllus, *Dascyllus trimaculatus* (Perciformes: Pomacentridae). *Zoological Studies* **43**(4): 803-812. The primary structure of the *Dascyllus trimaculatus* mitochondrial (mt) control region was determined, and conserved features were identified based on sequence comparisons to other teleostean fish species. Three termination-associated sequence (TAS) motifs, a central conserved sequence block (CSB), and a pyrimidine tract were identified, indicating that the organization of the mt control region in D. *trimaculatus* is similar to those reported for other teleosts. However, 2 sets of variable number of tandem repeats (VNTRs), denoted R1 and R2, which simultaneously occur at both the 5'- and 3'-ends of the mt control region are first reported herein in teleost fish. Analysis of amplification of the VNTRs indicated that heteroplasmy is common for both sets of VNTRs. VNTRs also respond to sequence length variation of the *D. trimaculatus* mt control region. A relatively high proportion of VNTR copy number variations and heteroplasmy indicate the potential utility of VNTRs as markers for assessing three-spot dascyllus population-level variability. http://www.sinica.edu.tw/zool/zoolstud/43.4/803.pdf

Key words: Three-spot dascyllus, Mitochondrial control region, VNTR, Heteroplasmy, Teleost.

The mitochondrial (mt) control region serves as the only non-coding sequence of the fish mt genome (Rand et al. 1993, Lee et al. 1995). It is composed of a central conserved sequence block (CSB) that is flanked by highly variable right and left domains (reviewed in Brown et al. 1986, Moritz et al. 1987, Rand 1993). The CSB contains a conserved sequence block-D (CSB-D) that is involved in heavy-strand replication, including initiation by a 3-stranddisplacement (d-loop, Clayton 1982). The right domain contains the site of origin for heavy-strand replication (O_H) and the normally conserved sequence blocks (CSB 1, 2, and 3) that are thought to function in its initiation (reviewed in Clayton 1982). The left domain, on the other hand, usually has 1 or more copies of the termination-associated sequence (TAS, Clayton 1982) that signals termination of d-loop strands (Doda et al. 1981, Lee et al. 1995).

In addition to the conserved characteristics, the mt control region also contains highly polymorphic sequences in the left and right domains that have made them the most-popular markers for addressing evolutionary relationships among populations and species (reviewed in Palumbi 1996). Nucleotide polymorphisms (point mutations) and

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variable numbers of copies of tandemly repeated sequences (VNTRs) are the 2 most-common types of sequence variations in the mt control region (Lee et al. 1995, Cesaroni et al. 1997, Stepien and Kocher 1997, Ravgo et al. 2002). Nucleotide polymorphisms in the mt control region have been intensively used to distinguish among species and population structures of fishes (Bermingham et al. 1986, McMillian and Palumbi 1995, Shulman and Berminghan 1995, Turner 1997, Dudgeon et al. 2000, Bernardi et al. 2001 2002, Chen et al. 2004).

On the contrary, the utility of VNTRs found in the mt control region in evolutionary studies has not been explored, probably due to their mechanisms of mutational models (Rand and Harrison 1989, Buroker et al. 1990, Madsen et al. 1993, Broughton and Dowling 1994, Brown et al. 1996, Mundy et al. 1996, Wilkinson et al. 1997, reviewed in Lunt et al. 1998), and heteroplasmy (intra-individual and intraspecific length polymorphisms) caused by the existence of VNTRs (Lee et al. 1995, Lunt et al. 1998). VNTRs are typically located in the left domain of the control region flanking the proline tRNA (*trnP*) gene in teleosts, but are also found in the right conserved domain beyond CSB 2-3 blocks in plaice, yellowtail, and salmon (Lee et al. 1995, Faber and Stepien 1998, Nesbo et al. 1998, Ludwig et al. 2000, Ravago et al. 2002). Nevertheless, the co-occurrence of 2 sets of VNTRs at both domains of the mt control region has not previously been reported in fish. Although VNTRs have provided reliable phylogenies in some studies (white sturgeon, *Acipenser transmontanus*, Brown et al. 1992), caution when applying this type of marker in phylogenetic and population genetic studies has been recommended (reviewed in Lunt et al. 1998).

The three-spot dascyllus, *Dascyllus trimaculatus*, is a damselfish widely distributed across the Indo-Pacific (Allen 1991, Godwin 1995). This species has been used as a representative species in ecological, behavioral, and genetic studies of coral reef fishes (Schmitt and Holbrook 1996 1999b, Bernardi and Crane 1999, Bernardi et al. 2001 2002). Direct sequencing of PCR products of the 5'-end of the mt control region for population comparisons has revealed the existence of a VNTR in *D. trimaculatus*, but no heteroplasmic individuals were found (Bernardi et al. 2001 2002). The purpose of the present study was to understand the structure and organization of the complete DNA sequence of the *D. trimaculatus* mt control region derived from PCR-amplified products spanning the cytochrome *b* (cytb) to the 12S ribosomal RNA (rRNA) genes. Our data demonstrate that characterization of the *D. trimaculatus* mt control region is unusual in teleost fish in that it contains 2 sets of VNTRs, R1 and R2, at the left and right domains, respectively. R1 (23 bp) begins 28 bp downstream of the *trnP* gene at the left domain which was reported in previous studies (Bernardi et al. 2001 2002). R2 (105 bp) is located 9 bp upstream of the phenaline tRNA (*trnF*) gene. Cloning and sequencing of the PCR products suggest the existence of heteroplasmy for both sets of VNTRs, and the presence of both VNTRs is responsible for the observed length polymorphism in the mt control region of *D. trimaculatus*. The utility of mt VNTRs for population genetic studies of *D. trimaculatus* in the South China Sea is highlighted.

MATERIALS AND METHODS

Fish samples

Fish collections were obtained through an international collaborative project among 6 countries around the South China Sea and the Solomon Is., coordinated by the WorldFish Centre in 1997. Detailed sampling localities were described in Chen et al. (2004). Fish were frozen whole in liquid nitrogen and shipped to the headquarters of the WorldFish for allozyme electrophoresis analysis (Ablan et al. 2002). Subsequently, a piece of muscle tissue was removed, preserved in 95% ethanol, and shipped to the laboratory of the senior author at the Institute of Zoology, Academia Sinica, Taiwan for DNA analysis. In total, 36 individuals were used in this study.

DNA extraction, amplification, cloning, and sequencing

DNA extraction was modified from methods described by Chen and Yu (2000), Chen et al. (2000), and Chen et al. (2004). The region spanning the 3'-end of the cytochrome *b* (cytb) gene and the 5'-end of the 12S rRNA gene was amplified using primers CB3R-5 and 12SAR-3 (Table 1) originally described by Palumbi (1996). PCR was performed in a PC-9606 thermal sequencer (Corbett Research, Mortlake, Australia) using the following thermal cycle: 1 cycle at 95°C (4 min); 4 cycles at 94°C (30 s), 50°C (1 min), and 72°C (2 min); and 35 cycles at 94°C (30 s), 55°C (1 min),

and 72°C (2 min). The amplification reaction used 50~200 ng of template and BRL *Taq* polymerase in a 50-µl volume reaction with the buffer supplied with the enzyme and under the conditions recommended by the manufacturer. The PCR products were electrophoresed in a 1% agarose (FMC Bioproduct, Rockland, ME, USA) gel and 1X TAE buffer to check the yield. The amplified DNA was extracted using a DNA/RNA gel extraction column (Viogene, Taipei, Taiwan) under conditions recommended by the manufacturer. PCR products were then cloned using the pGEM-T system (Promega, Madison, WI, USA) under conditions recommended by the manufacturer. The nucleotide sequences were determined for both light (L) and heavy (H) strands by a primer walking strategy using an ABI 377 genetic analyzer. The internal primers designed for DNA sequencing, and their orientation and positions located in the mt control region are listed in table 1 and figure 1, respectively. The sequences obtained in this study were submitted to GenBank under accession numbers AY751750.

Sequence analysis

Nucleotide sequences of amplified fragments of *Dascyllus trimaculatus* were initially aligned using CLUSTAL W 1.7 (Thompson et al. 1994), followed by manual editing using SeqApp 1.9 (Gilbert 1994). Putative gene regions (cytb, transfer RNA (tRNA), control, and 12S rRNA), conserved sequence blocks (CSBs), andstructures were localized by alignment with sequences from fish available in GenBank. The identified CSBs were then compared to conserved features of other vertebrates, including the human, mouse, and frog tRNA sequences and were also confirmed using a tRNAscan-SE (Lowe and Eddy 1997).

RESULTS AND DISCUSSION

We determined the complete nucleotide sequence of the *Dascyllus trimaculatus* mitochondrial (mt) control region, and 3 tRNA genes, as well as partial sequences of the flanking region, cytb, and 12S rRNA. Lengths of PCR fragments ranged from 2383 to 2587 bp, including 320 bp for cytb, 420 bp for 12S rRNA, 72 bp for *trnT*, 69 bp for *trnP*, and 70 bp for *trnF*. The total length of the control region was identified to range from 1428 to 1632 bp in *D. trimaculatus*. This size range is comparable to the control regions of other teleosts (Cecconi et al. 1995, Lee et al. 1995, Lunt et al. 1998, Ravago et al. 2002). The length of the *D. trimaculatus* mt control region is similar to those in plaice (1553 bp) and yellowtail (~1600 bp), but much longer than those in a cichild (888 bp), Atlantic cod (996 bp), salmon (1071 bp), and sturgeon (976 bp) (Lee et al. 1995). The observed length variability of the *D. trimaculatus* mt control region is due to the presence of different copy numbers of the repeat units, R1 and R2, in both ends of the control region (see below). Localization of the putative gene regions and the overall structure of the control region are shown in figure 1.

(a) Conserved sequences and homology to other fish mt control regions

The general organization of the mt control

Table 1. Primers used in mtDNA amplification and sequencing of *Dascyllus trimaculatus*

Primer	Position	Oligonucleotide sequence
CB3R-5[a]	Cytochrome b	L[b], 5'- CAY ATY MAR CCM GAA TGR TAY TT -3'
tRNA-5	tRNA-pro	L, 5'- GCT YAG TMT BAR AGC RYC GGT CTT G-3'
3-internal-5	D-loop	L, 5'- CAG AAA TCA TAA GGG TTG C- 3'
3spJ1new	D-loop	L, 5'- GCA TTT GGG TTC CTA TTT CAG GGC C- 3'
3spJ1two	D-loop	L, 5'- CTG GCA THT GGT TCC TAY TTC AGG KC- 3'
J1R	D-loop	H[b], 5'- AMA ATK TAT GGC CCT GAA ATA GGA ACC- 3'
12SV1	12S rRNA	H, 5'- CAT YCT CAC CGG GSY GCG GAK ACT YGC-3'
12 SAR-3[a]	12S rRNA	H, 5'- ATA GTG GGG TAT CTA ATC CCA GTT -3'

[a]Primers from Palumbi (1996); [b] L: light strand; H: heavy strand.

region in *D. trimaculatus* (Fig. 2) is similar to that reported for other teleosts (Broughton and Dowling 1994, Lee et al. 1995, Nesbo et al. 1998, Ravago et al. 2002). Three termination-associated sequence (TAS) motifs, proposed to act as a sequence-specific signal for the termination of D-loop synthesis (Doda 1981), have been identified as being 30 bp downstream of the 1st repeat unit (R1) in the 5' peripheral domain, and these show high similarity with other putative TAS elements in other fish species (Table 2). A central conserved section was found in the downstream 3' direction that includes the conserved sequence block-D (CSB-D), which is apparently highly conserved in fish (Lee et al. 1995, Faber and Stepien 1998, Ravago et al. 2002). Although the function of this central conserved region is not well understood, it is the most universally conserved segment of CSB-D among fish families, suggesting that it possesses functions critical for mitochondrial metabolism. A motif, GACATA, identical to conserved sequence block 1 (CSB-1) of the seabass, *Dicentrarchus labrax* (Cecconi et al. 1995), was found, although CSB-1 is less conserved and is difficult to unambiguously identify in teleost fishes (Lee et al. 1995). Additional conserved sequence blocks 2 and 3 (CSB-2 and 3) were identified at the right domain near the 2nd repeat unit (R2) (Table 3). The pyrimidine tract (PY) and the origin of the H-strand replication (O_H) located between CSB-D and CSB-1 were identified based on their similarity to those of other teleosts (Fig. 2).

(b) Variable number of tandem repeats (VNTRs) and heteroplasmy

The mt control region of *D. trimaculatus* is unusual in teleost fishes in that it contains 2 sets of tandem repeats located at both the 5'- and 3'-ends of the control region. Sequence analysis indicates the presence of a 23-bp tandem repeat (R1), identical to that of previous reports (Bernardi et al. 2001 2002), which is located at the 5'-end of the control region 28 bp downstream of the *trnP* gene (Figs. 1, 2), where replication of the D-loop is terminated (Lee et al. 1995). Specific primers, tRNA-5 and J1R (Table 1), were designed to amplify the region containing R1 (Fig. 3). A survey of 36 individuals from different localities in the South China Sea indicated that the shortest sequence (197 bases) contained 1 copy of R1, and the longest

Table 2. Putative TAS elements from *Dascyllus trimaculatus* and other fishes

Species	DNA sequence	TAS
Dascyllus trimaculatus	5'-ACTATGTATGTACTT	1
	5'-ACATAAGCTTAAGTT	2
	5'-CCATAAACCTTGTAAG	3
Chanos chanos [a]	5'-ACATACTATGT	1
Stizostedion spp. [b]	5'-ACATCTATATTAACT	1
	5'-ACATATATGTTTT	2
	5'-ACATTCATATATCCC	3
	5'-ACATAAAGCAT	4
Dicentrarchus labrax [c]	5'-ACATGATATGT	1
	5'-ACATATTATGT	2
	5'-ACATATCATGT	3
	5'-GCATATTATGT	4
Crossostoma lacustre [d]	5'-ACATATTATGC	1
	5'-ACATTACTATGT	2
	5'-ACATTACTATGT	2
Gadus morhua [e]	5'-TCATAATATGC	1
	5'-ACATATGTATAAT	2
Salmo trutta [f]	5'-ACATATTATGT	1
	5'-ACATCATATGT	2

References: [a] Ravago et al. (2002); [b] Faber and Stepien (1998); [c] Cecconi et al. (1995); [d] Tzeng et al. (1992); [e] Chang et al. (1994); [f] Bernatchez et al. (1992).

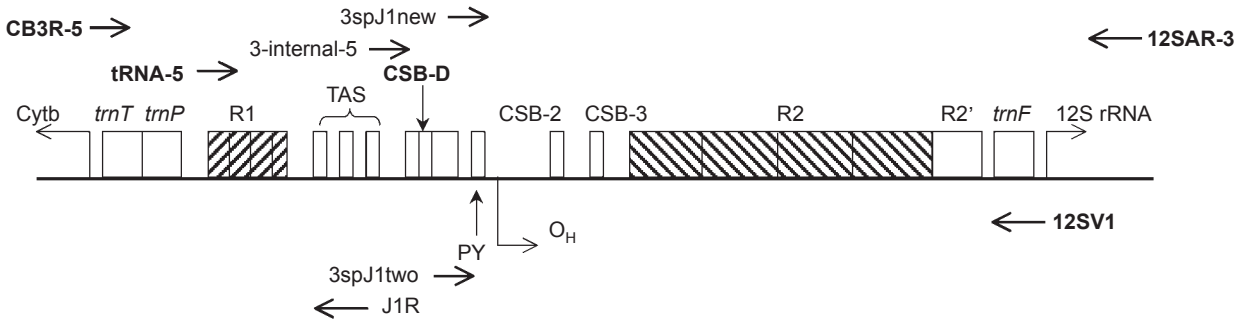

Fig. 1. Schematic diagram of the structure and organization of the mitochondrial control region (L strand) of the three-spot dascyllus, *Dascyllus trimaculatus*. Conserved sequence elements and tRNAs are indicated by boxes. Primer positions are indicated by arrows. Sequences of primers are listed in table 1.

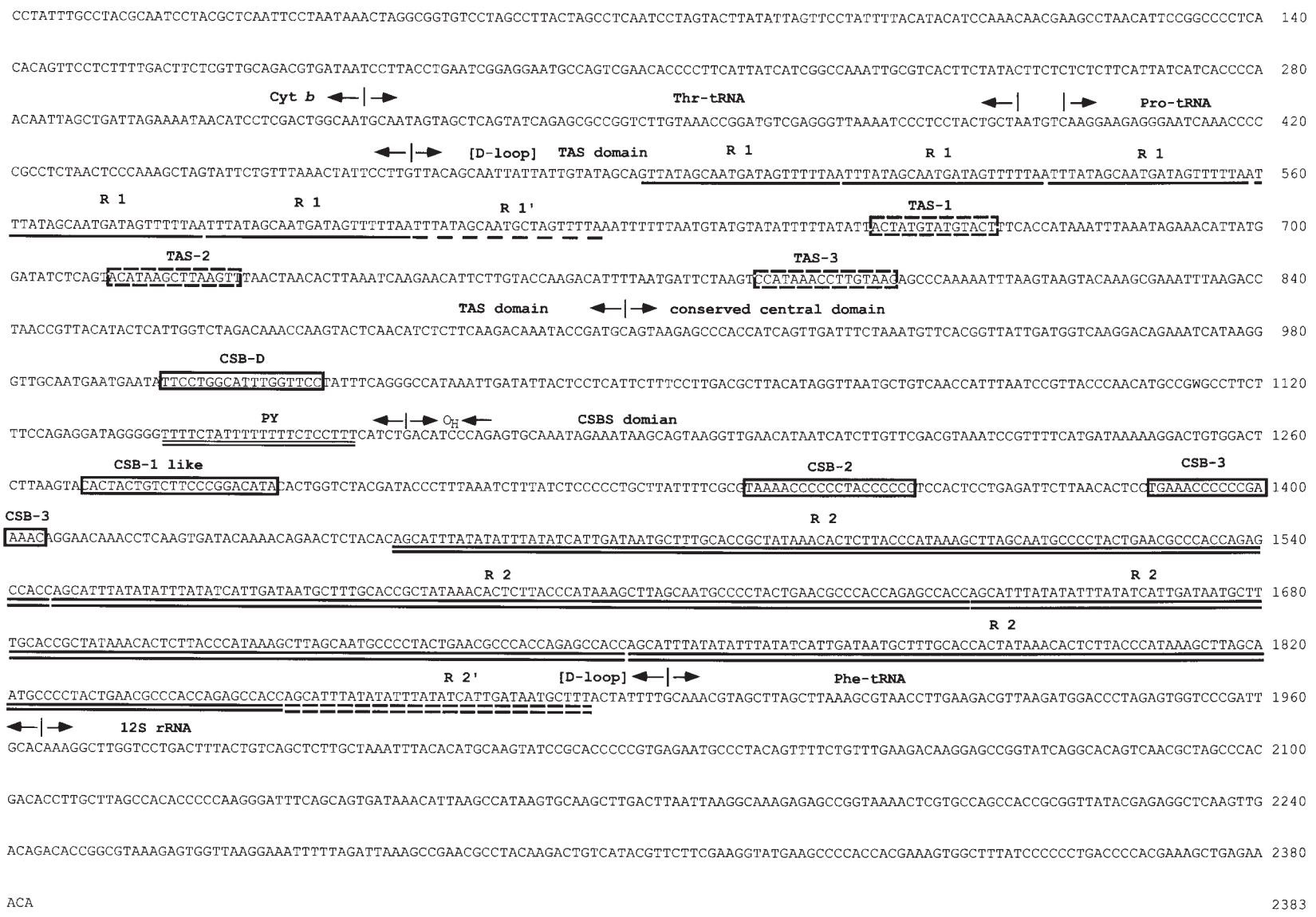

Fig. 2. The dascyllus mtDNA control region, including the flanking cytochrome b, 12S rRNA, and tRNA . The 1st (R1) and 2nd (R2) tandem repeats are underlined and double-underlined, respectively. Termination-associated sequences (TASs) and conserved sequence block (CSB) motifs are boxed. This sequence has been submitted to GenBank under accession no. AY751750.

Table 3. Comparison of conserved sequence elements in the *Dascyllus trimaculatus* mt control region to elements in other fish species

Species	CSB-D	CSB-I	CSB-II	CSB-III
Dascyllus trimaculatus [a]	5'-TTCCTGGCATTTGGTTCC	5'-CACTACTGTCTTCCCGGACATACA	5'-TAAAACCCCCCTACCCCCCT	5'-TGAAACCCCCCGAAAACA
Chanos chanos [b]	5'-TTCAAGAGCATCTGGTTCC	-	5'-AAACCCCCCCCTCCCCCCA	5'-TGTTAAACCCCCAAAACCA
Perca fluviatilis [c]	5'-TTCCTGGCATTTGGTTCC	5'-ATCTTAGGATATCAAGAGCATAA	5'-TAAACCCCCCCTACCCCCCC	5'-TGAAAACCCCCCCGGAAACA
Acerina cernua [c]	5'-TTCCTGGCATTTGGTTCC	5'-ATCTTAGGATATCAAGAGCATAA	5'-TAAACCCCCCCTACCCCCCC	5'-TGAAAACCCCCCCGGAAACA
Stizostedion lucipoerca [c]	5'-TTCCTGGCATTTGGTTCC	5'-ATCTTAGGATATCAAGAGCATAA	5'-TAAACCCCCCCTACCCCCCC	5'-TGAAAACCCCCCCGGAAACA
Dicetrarchus labrax [d]	-	5'-TTTATCGTAAGTGACATAGGTAA	5'-AATTTCCCCCCCTACCCCCCC	5'-TGCTCAAAATAAATCCCCCTAAGAAAAAGCA
Crossostoma lacustre [e]	5'-TTACTGGCATCTGGTTCC	5'-CTATGTATGTAGAATGAGCATAA	5'-ACAAACCCCCCTACCCCCCT	5'-TGCTCAAACCCCGAAACCA
Gadus morhua [f]	-	5'-ATTAAAGTTTTTCAAGAGCATAA	-	-
Salmo trutta [g]	-	5'-ATACTTGGATATCAAGTGCATAA	-	-

References: [a] this study; [b] Ravago et al.(2002); [c] Nesbo et al.(1998); [d] Cecconi et al.(1995); [e] Tseng et al.(1992); [f] Johansen et al.(1990); [g] Bernatchez et al.(1992); -: not identified.

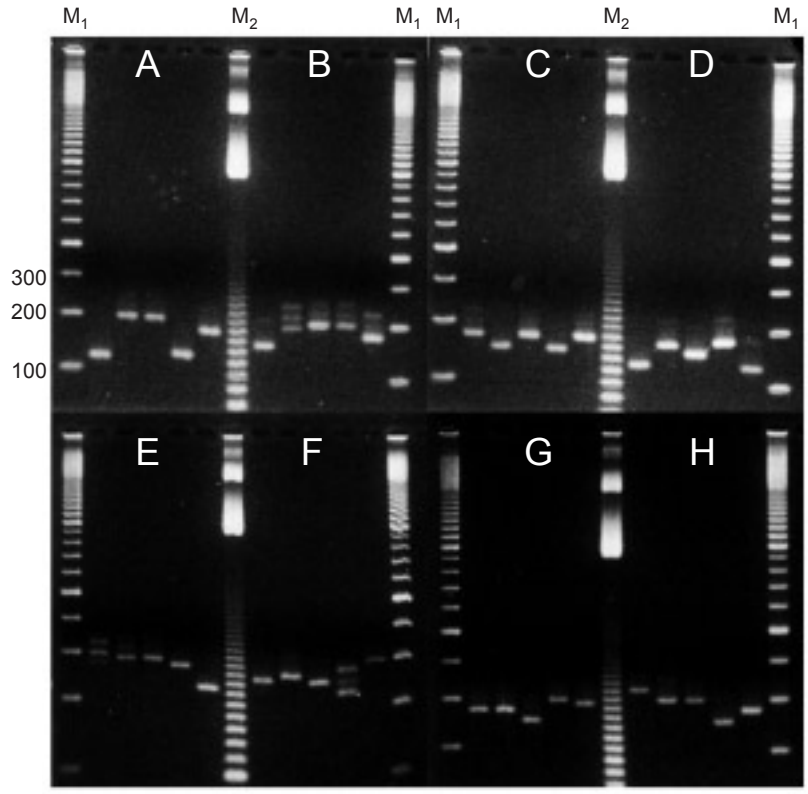

Fig. 3. Three-spot dascyllus mtDNA length variants of the 1st VNTR (R1) and its heteroplasmy detected by the polymerase chain reaction. Five individuals per populations were used. A: northern Taiwan and B: the Penghu Is., Taiwan; C: Batangas and D: Cebu, the Philippines; E: Paulau Tiga and F: Paulau Usukan, Malaysia; and G: Na Trang and I: Ca Na Bay, Vietnam. M1: 100-bp molecular ladder; M2: 20-bp molecular ladder.

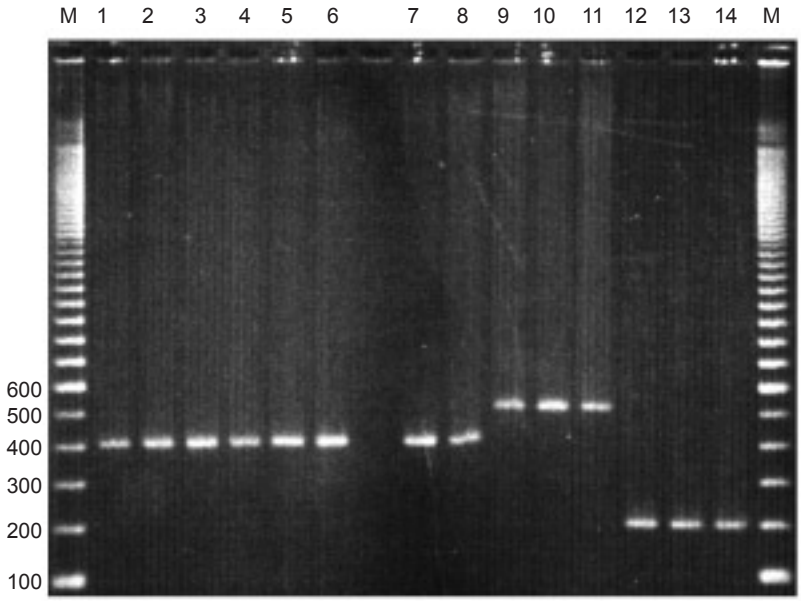

Fig. 4. Three-spot dascyllus mtDNA length variants of the 2nd VNTR (R2) and its heteroplasmy detected by cloning and the polymerase chain reaction. Lanes 1-3: clones derived from 1 individual sample collected from northern Taiwan; lanes 2-6: clones derived from 1 individual sample collected from the Penghu Is., Taiwan; lanes 7-11: clones derived from 1 individual sample collected from Cebu, the Philippines; lanes 12-14: clones derived from 1 individual sample collected from Gela, the Solomon Is. M: 100-bp molecular ladder.

sequence (404 bases) contained 10 copies (Fig. 5). The most frequently observed copy number of R1 was 6 among the 36 individual fishes surveyed. This is similar to the finding in a study of species boundaries and color patterns of the *D. trimaculatus* complex (Bernardi et al. 2002). The 2nd tandem repeat, denoted R2 (105 bp), is located 9 bp upstream of the phenaline tRNA (*trnF*) gene (Figs. 1, 2). PCR and sequencing analysis using the set of specific primers, 3spJ12 and 12 SV1, indicated that the copy number of R2 ranged from 1 to 6 copies (Fig. 4). R2 has an imperfect R2' repeat at the junction with *trnF* (Fig. 1).

Two characteristics of the mt control region VNTR in *D. trimaculatus* are unique. First, 2 sets of VNTRs simultaneously located at both the 5'- and 3'-ends of the mt control region have not been observed in other fish. Second, R2 (105 bp) is the 2nd-longest VNTR unit reported in teleost fish, only shorter than that (260 bp) of minnows (reviewed in Lunt and Wipple 1998). Lee et al. (1995) studied the control region of 23 species of fish using a strategy of PCR amplification with various primers targeted to conserved sequences in the middle of the control region and to the tRNA gene on either side. They reported VNTRs in a number of species, including plaice (61 bp), yellowtail flounder (64 bp), Atlantic cod (40 bp), salmon (20 bp), and sturgeon (82 bp). Among these, the VNTRs of plaice, yellowtail flounder, and salmon were located on the right side of CSB2-3, which is toward the 3'-end of the mt control region. In contrast, in several fish species, i.e., Atlantic cod, several species of sturgeon (Brown et al. 1992, Ludwig et al. 2000), European sea bass (Cecconi et al. 1995), and milkfish (Ravago et al. 2002), the VNTR is located at the 5'-end of the control region flanking the *trnP* gene.

The presence of multiple PCR products from individual templates despite extensive optimization of amplification conditions is suggestive of heteroplasmy, where multiple length variants are found within an individual. An initial screening of length variations of R1 by agarose gel electrophoresis (Fig. 3) and sequencing of the cloned PCR fragments revealed ~58% heteroplasmy, with up to 6 length variants observed within an individual (data not shown). Length variants of R2 were also observed within an individual after cloned PCR fragments were reamplified and run on agarose electrophoresis (Fig. 4). Our results are in contrast to a previous population study of *D. trimaculatus* which detected no heteroplasmy in R1 repeats (Bernardi et al. 2001 2002). Length variants caused by VNTRs can be an artifact of the slippage effect by *Taq* polymerase during PCR amplification. It is difficult to distinguish between *Taq* slippage and actual heteroplasmy, if the 2 bands differ by only 1 repeat. However, 2 bands that differed by more than 1 repeat unit with nothing between them in a single individual were observed in our study (e.g., MPU2, PP1, and VQ3 in Fig. 5), supporting the occurrence of heteroplasmy in the mt control region of *D. trimaculatus*. In many teleosts, the mitochondrial region is characterized by VNTRs and a high level of heteroplasmy (Nesbo et al. 1998, Ludwig et al. 2000). The causes of variation in the repeat number and of heteroplasmy are not fully understood, but strand slippage (Levinson and Gutman 1987), intra- and intermolecular recombination (Rand and Harrison 1989), and unequal crossing-over or gene conversion (Hoelzel et al. 1995) have been proposed to account for the maintenance of length variability and heteroplasmy of mt VNTRs. Heteroplasmy may also be related to mtDNA recombination, although it is difficult to demonstrate this mechanism (Hoarau et al. 2002, reviewed in Rokas et al. 2003).

(c) VNTRs as potential markers for population genetic studies in *D. trimaculatus*

PCR amplification of R1 using a specific primer set demonstrated the utility of mt VNTRs in the population genetic analysis of *D. trimaculatus* in the South China Sea (Fig. 3). Length differences of PCR products could easily be observed within and among the 8 populations from Taiwan (2), the Philippines (2), Vietnam (2), and Malaysia (2). In addition, the proportion of individuals with heteroplasmy also differed among populations. Such differences can provide information for a hierarchical statistical analysis of heteroplasmic data in order to reveal population subdivisions (Birky et al. 1983, Rand and Harrison 1989). Caution has been urged when applying mt VNTR to population differentiation and biogeography, although mt VNTR analysis, once detected by PCR, can provide a sensitive, rapid, and cost-effective measure of genetic variability (reviewed in Lunt et al. 1998). One of the concerns is the reliability of the detection procedure of alleles and the scoring of intra-individual allele frequencies. In preliminary tests of the R1 VNTR variation of *D. trimaculatus*, PCR products were visualized using 2% EtBr-stained agarose gels. Only weaker bands were detected in heteroplasmic individuals,

suggesting that allelic numbers of heteroplasmic individuals might be underestimated if the yields of PCR products are poor (Fig. 3). Directly incorporating isotope labeling into PCR reactions and separating alleles using polyacrylamide sequencing gels can improve the scoring of intra-individual allelic frequencies and provide consistent results (Chen et al. unpubl. data).

In summary, the complete DNA sequence of the mitochondrial control region was determined for the three-spot dascyllus, *Dascyllus trimaculatus*. Three TAS motifs, a central conserved sequence block, and a pyrimidine tract were identified, indicating that the organization of the mt control region in *D. trimaculatus* is similar to those reported for other teleostean fish. The simultaneous occurrence of 2 sets of VNTRs (R1 and R2) at both the 5'- and 3'-ends of the mt control region of a teleostean fish is first reported herein. Analysis of the amplification of VNTRs indicated that heteroplasmy is common for both sets of VNTRs. Furthermore, it should be possible to conduct PCR amplification of the R1 region with relative ease. Thus with careful consideration of theoretical and interpretive factors, this VNTR is potentially useful as a molecular marker for detecting and assessing population-level variability of the three-spot dascyllus.

Acknowledgments: Many thanks to S.Y. Rau and T.C. Hsui for laboratory work, and the staff of the Penghu Aquarium, a facility of the Taiwan Fishery Research Institute, for collecting fish specimens from the Penghu Is. We thank members of the Evolution and Ecology Discussion Group, Institute of Zoology, Academia Sinica (IZAS), for constructive comments before submission, and 2 anonymous reviewers for the revision. This work was supported by grants from the John D. and Catherine T. MacArthur Foundation to the PISCES project through the WorldFish Centre, and IZAS internal grants to C.A.C and K.T.S. This is Evolution and Ecology Group, IZAS contribution no. 25.

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