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Universal Primers for Amplification of the Complete Mitochondrial 12S rRNA Gene in Vertebrates

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Hurng-Yi Wang, Mung-Pei Tsai, Ming-Chung Tu and Sin-Che Lee (2000) Universal primers for amplification of the complete mitochondrial 12S rRNA gene in vertebrates. *Zoological Studies* **39**(1): 61-66. The conserved regions of *tRNA^{PHE}* and *16S rRNA* in the vertebrate mitochondrial genome were compared in order to design the primers, 12SR and 12SL. These universal primers can be broadly used to amplify a 1.3-kb DNA fragment by polymerase chain reaction (PCR) over a wide range of major vertebrate lineages represented by the species listed in the text. There is little length variation of the PCR product among different taxa. Further sequence analysis revealed that the fragment contains complete lengths of *12S rRNA* and *tRNA^{VAL}*, and that the length of *16S rRNA* is 200 bp. In tests through all representative taxa investigated, the above 2 primers could amplify the complete 12S rRNA gene from all representative taxa investigated. As the 12S rRNA gene is widely used for phylogenetic analyses among different hierarchies, the use of these primer sets for study of higher-category phylogenies in vertebrates now becomes possible.

Key words: 12SR and 12SL, Primers, PCR amplification, Vertebrates.

Molecular markers are used as tools for estimating the phylogenetic relationships of different kinds of organisms (Avise 1994). Although various techniques, such as allozyme analysis, RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), and mini- and microsatellites, have been employed for molecular systematics, most investigators are examining DNA sequence polymorphism, which is the most fundamental unit of molecular variation. So it is important to choose an appropriate genetic marker for phylogenetic analysis. Ribosomal RNA (rRNA) genes, including 4 nuclear rRNA genes and 2 mitochondrial rRNA genes, are some of the most widely used genetic markers for phylogenetic analyses. The mitochondrial rRNA genes, including *12S* and *16S*, evolve much more rapidly than the nuclear rRNA genes. As *rRNAs* (both among and within genes) evolve at different rates, *rRNA* sequences have been used to infer phylogenies across a very broad spectrum, from studies among the lineages of life to relationships among closely related species and populations (Hillis and Dixon 1991).

Of the rRNA genes, *12S mitochondrial rRNA* has been widely used to study the phylogenetic relationships among different levels of taxa such as families (Alves-Gomes et al. 1995, Douzery and Catzeflis 1995, Ledje and Arnason 1996), genera (Gatesy et al. 1997, Murphy and Collier 1997), and species (Murphy and Collier 1996, Halanych and Robinson 1997). Because of the broad spectrum of phylogenetic analyses of *12S rRNA*, especially in vertebrates, using PCR to amplify the complete region of this gene for further analyses will be very useful. As reported in this paper, we designed primer pairs based on sequences of conserved regions of *tRNA^{PHE}* and *16S rRNA* of the mitochondrial genome from GenBank, and used them to amplify the complete 12S rRNA and partial 16S rRNA genes from major lineages of vertebrates.

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MATERIALS AND METHODS

Primer design

Published mitochondrial DNA sequence of *tRNA^{PHE}* and of the 5' end of *16S rRNA* from fish (*Crossostoma lacustre*; Tzeng et al. 1992), frog (*Xenopus laevis*; Roe et al. 1985), chicken (*Gallus gallus*; Desjardins and Morais 1990), and lizard (*Sceloporus undulatus*; Hedges 1994) or green turtle (*Chelonia mydas*, Kumazawa and Nishida 1999) were aligned (Fig. 1). Two primers (12SR: 5'-TTT CAT GTT TCC TTG CGG TAC-3'; 12SL: 5'-AAA GCA CGG CAC TGA AGA TGC-3') were designed from their conserved regions: 12SL was from the 21st to 41st bases of *tRNA^{PHE}*, while 12SR was from the 113th to 133rd bases of *16S rRNA*.

DNA amplification and sequencing

The samples used for DNA studies are listed in table 1. All samples were collected from Taiwan or the waters around Taiwan. With the exceptions of extracted DNA samples of human leukocytes provided by Dr. Guey-Jen Lee-Chen and extracted DNAs from muscle of *Eumeces elegans* and *Hynobius formosanus* provided by Dr. Kuang-Yang Lue, the DNAs of remaining samples were extracted from muscular tissues in our laboratory.

Extraction of crude DNA from fresh or 95% ethanol-preserved muscle samples followed the standard procedures of Kocher et al. (1989). PCR was performed in a 50-µl reaction which consisted of 5 µl of 10X reaction buffer (10 mM Tris HCl, pH 9.0; 50 mM KCl; 15 mM $MgCl_2$; 0.1% [w/v] gelatin; 1% Triton X-100), 0.4 µM each of the primers, 0.2 µM each of dNTP, 50-100 ng crude DNA, and 1 unit *Taq* DNA polymerase (Gibco BRL, Life Technologies Inc., NT, USA). In order to determine the annealing temperature of PCR using our designed primers, DNA of *Egretta garzetta* was processed in a Stratagene thermal cycler with a gradient program through 35 cycles under the following conditions: initial denaturation for 1 min at 94 °C, primer annealing for 1 min at 46 to 54 °C by 1 degree intervals, extension for 1.5 min at 72 °C, followed by a final extension for 10 min at 72 °C. Once the experimental annealing temperature was selected, the subsequent conditions for PCR in other taxa were carried out accordingly. The PCR products were eluted using a Gene Clean III elution kit (Bio 101 Inc., La Jolla, USA), following the instructions of the manufacturer, and were directly sequenced. DNA sequences were determined using dye terminator cycle sequencing reactions that were subsequently loaded on an Applied Biosystems 377A automatic sequencer, following the manufacturer's protocols (Perkin Elmer Cetus, Norwalk, USA).

Sequences were aligned using the PILEUP program of the Genetics Computer Group (GCG) Package, version 7.0 (Devereux et al. 1991), and were adjusted visually. Aligned sequences were analyzed using the MEGA software package (Molecular Evolutionary Genetics Analysis, version 1.02; Kumar et al. 1993) for calculating nucleotide substitutions between sequences.

RESULTS AND DISCUSSION

The primers were successfully used to amplify an expected DNA fragment of about 1.3 kb at all temperature regimes performed using the DNA of *Egretta garzetta* as a template (Fig. 2A). The PCR

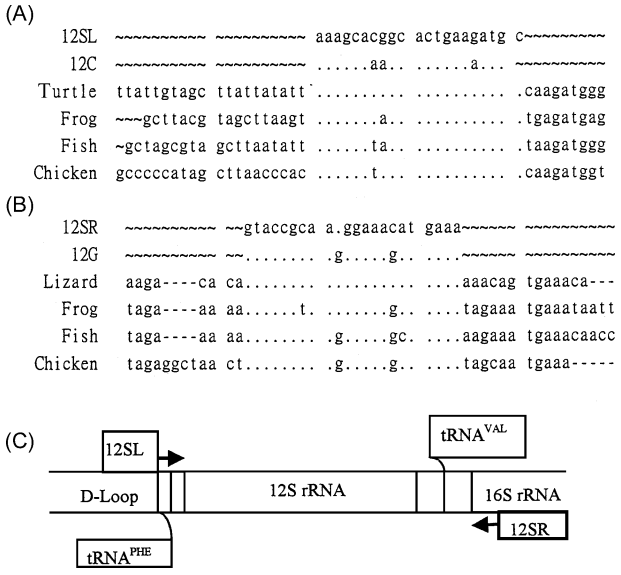

Fig. 1. Sequences of 12SL and 12SR primer pairs and their positions relative to the mitochodrial genes. (A) Sequence of 12SL primer, derived from a conserved region of the tRNA^{PHE} gene compared with primer 12C (Springer et al. 1995), green turtle (Kumazawa and Nishida 1999), frog (Roe et al. 1985), fish (Tzeng et al. 1992) and chicken (Desjardins and Morais 1990). The tilde, ~, indicates an empty base position. Dots indicate matches with other sequences. Dashes denote gaps. (B) Sequence of 12SR primer, derived from a conserved region of the 16S rRNA gene, and that compared with primer 12G (Springer et al. 1995), frog (Roe et al. 1985), fish (Tzeng et al. 1992), lizard (Hedges 1994), and chicken (Desjardins and Morais 1990). (C) Partial mitochodrial genes and the relative positions of the rRNA genes (*12S rRNA* and *16S rRNA*), tRNA genes (*tRNA^{PHE}* and *tRNA^{VAL}*), and D-loop. Arrows indicate locations and directions of primers used in this study.

product yielded a subband when the annealing temperature was lower than 50 °C. Figure 2A indicates the single product of PCR when 50 °C was used as the annealing temperature. The DNAs extracted from the samples in table 1 were used as templates, from which a DNA fragment of 1.3 kb was successfully amplified (Fig. 2B). PCR products showed little length variation among more primitive species, or even higher taxonomic levels. Comparing the sequences of the complete PCR products of *Sphyrna zygaena, Terapon jarbua, Scomber japonicus,* and *Trimeresurus mucrosquamatus* with other published sequences in GenBank, that the amplified DNA fragment was recognized to contain the complete 12S rRNA and $tRNA^{VAL}$ genes, as well as partial 16S rRNA genes of about 200 bp in length. The nucleotide composition of the complete *12S rRNA* among different groups is quite similar, with average percentages of 33.5% (A), 25.5% (C), 19.1% (G), and 22.0% (T). The ratio of transitions and transversions based on pairwise comparisons varied from 0.575 to 1.078. The similarity among sequences of different groups ranged from 53.0% to 80.6%. *Alopias vulpinus, Pelates quadrilineatus, Terapon theraps, Pomadasys kaakan, Hynobius formosanus, Eumeces elegans, Egretta garzetta, Nycticorax nycticorax, Lepus sinensis formosus,* and *Homo sapiens* were partially sequenced, and their PCR products did contain the 12S rRNA gene with a consistency of gene order among the species. We subsequently used the above 2 universal primers to amplify the *12S rRNA* region of the thornfishes *Pelates quadrilineatus* (*n* = 6), *Terapon jarbua* (*n* = 6), and *T. theraps* (*n* = 6) as well as that of the grunt, *Pomadasys kaakan*

Table 1. Materials used in this study

Taxa			Species	Collecting Site
Class	**Chondrichthyes**			
Order	Lamniformes			
	Family	Alopiidae	*Alopias vulpinus*	Tahsi
Order	Carchariniformes			
	Family	Sphyrinidae	*Sphyrna zygaena*	Tahsi
Class	**Osteichthyes**			
Order	Perciformes			
	Family	Haemulidae	*Pomadasys kaakan*♦	Kaohsiung
		Teraponidae	*Pelates quadrilineatus*♦	Kaohsiung
			Terapon jarbua	Kaohsiung
			Terapon theraps♦	Kaohsiung
	Family	Scombridae	*Scomber japonicus*	Tahsi
Class	**Amphibia**			
Order	Urodela			
	Family	Hynobiidae	*Hynobius formosanus* (provided by Dr. Kuang-Yang Lue)	
Class	**Reptilia**			
Order	Squamata			
	Family	Scincidae	*Eumeces elegans* (provided by Dr. Kuang-Yang Lue)	
	Family	Viperidae	*Trimeresurus mucrosquamatus*	Taipei
Class	**Aves**			
Order	Ciconiiformes			
	Family	Ardeidae	*Egretta garzetta*	Taipei
			Nycticorax nycticorax	Taipei
Class	**Mammalia**			
Order	Primates			
	Family	Homonidae	*Homo sapiens* (provided by Dr. Guey-Jen Lee-Chen)	
Order	Lagomorpha			
	Family	Leporidae	*Lepus sinensis formosus*	Taipei

The voucher specimens have been deposited in the Museum of the Institute of Zoology, Academia Sinica.
♦The PCR products are not shown in figure 2B.

(n = 1). There was no intraspecific variation among 660 base pairs of sequences of the thornfishes aligned. The ratio of transitions (ts) and transversions (tv) between any 2 species from the nucleotide composition data was from 1.178 to 2.335. Pairwise comparisons of observed substitutions are similar, though slightly different among the 3 thornfish species. A dissimilarity of 15.34%-20.14% exists between the families Teraponidae represented by the above 3 species and Haemulidae represented by *Pomadasys kaakan*. Such DNA sequences are variable enough for investigation of phylogenetic relationships, because optimal similarities of DNA sequences for phylogenetic studies are between 70% and 100% (Hillis and Dixon 1991). The above data suggest that the *12S rRNA* sequences can be used for studies of phylogenic at the familial and generic levels. This might be due to the *12S rRNA* sequences which contain several regions having variable evolutionary rates, eg., the stems of the *12S rRNA* secondary structure evolved slower than did those of the loops.

The *12S rRNA* sequence can be used for implying phylogenic relationships across a broad spectrum, since transitional substitutions in the loops region have accumulated as fast as 10-20 my after the divergence event occurred. On the other hand, transversional substitutions in stems and loops remain unsaturated for more than 100 my (Springer and Douzery 1996), which enables the 12S rRNA gene to be more comprehensive for phylogenetic reconstruction. Having primers which can amplify the complete 12S rRNA gene of more animal groups would be helpful for phylogenetic studies. Our results indicate that our 2 primers can amplify the complete 12S rRNA gene from all represented taxa, since the species used in this study cover all major lineages of vertebrates. The 12S rRNA gene has been widely used in phylogenetic studies, while most primers used in previous studies would only amplify partial *12S rRNA* sequences in 1 or several particular vertebrate taxa. In addition, complete sequences allow implications of a more complete profile of gene evolution and provide insights into phylogenetic reconstructions that may not be possible with partial sequences (Springer et al. 1995). Therefore, primers that can amplify the complete 12S rRNA gene among vertebrates are useful tools for studying systematic and phylogenetic relationships.

Among the primer pairs designed for amplification of mammalian *12S rRNAs* (Springer et al. 1995), the sequences of 12C and 12G are similar to those of ours. However, 12C is 1 base shorter (C) at the 3' region, and has several bases different when compared with other vertebrates. Thus 12C is more specific for amplification of mammalian *12S rRNAs* rather than those of other vertebrates. On the other hand, 12SL matches well with the 5' region of *16S rRNA* of most vertebrates, which makes the primer work in more vertebrate groups once the *12S rRNAs* are amplified. Furthermore, we have tried 12C and 12G and have found they are unable to amplify the *12S rRNA* of some reptiles.

In many eukaryotic organisms, the integration of a mitochondrial gene into the nuclear genome has been frequently observed (Farrelly and Butow 1983, Fukuda et al. 1985, Lopez et al. 1994). It is important to identify the proper gene that we require through PCR. Another advantage of these primers is that the PCR fragment contains *12S rRNA, tRNA^{VAL}*, and *16S*

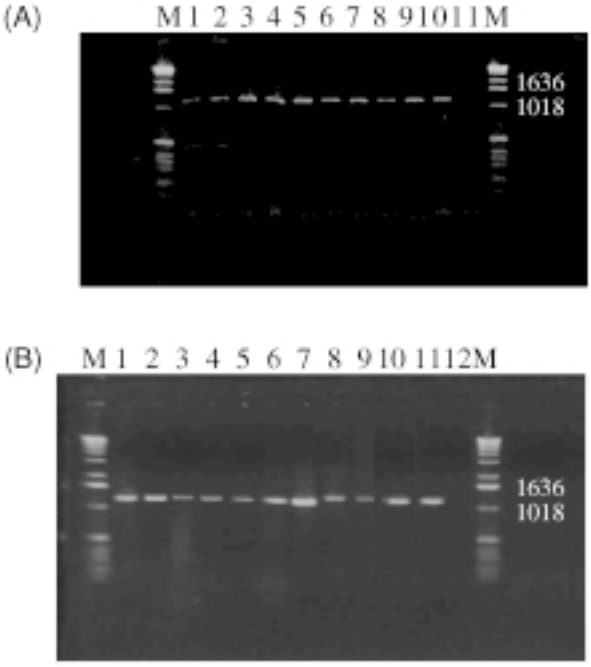

Fig. 2. (A) Expected size of DNA fragment of about 1.3 kb (between bands of 1 kb and 1.6 kb) amplified using the universal primers, at 46 to 55 °C as annealing temperatures performed by using the DNA of *Egretta garzetta* as a template. Lanes 1, 46 °C as annealing temperature; lane 2, 47 °C; lane 3, 48 °C; lane 4, 49 °C; lane 5, 50 °C; lane 6, 51 °C; lane 7, 52 °C; lane 8, 53 °C; lane 9, 54 °C; lane 10, 55 °C; lane 11, negative control at 50 °C as annealing temperature. (B) The DNA extracted from the samples in table 1 were used as templates and were successfully amplified. Lane 1, *Alopias vulpinus*; lane 2, *Sphyrna zygaena*; lane 3, *Terapon jarbua*; lane 4, *Scomber japonicus*; lane 5, *Hynobius formosanus*; lane 6, *Eumeces elegans*; lane 7, *Trimeresurus mucrosquamatus*; lane 8, *Egretta garzetta*; lane 9, *Nycticorax nycticorax*; lane 10, *Homo sapiens*; lane 11, *Lepus sinensis formosus*; lane 12, negative control. The columns on the outermost sides of the figure (M) are molecular weight markers.

rRNA of 200 base pairs in length. Such a gene order in the mitochondrial genome is found consistently throughout all vertebrates with a limited length variation. In addition, evolutionary rates of different regions of the DNA sequences are highly variable; for instance, the $tRNA^{VAL}$ and 3rd domain of 12S rRNA genes are relatively conserved when compared with other regions. If the mitochondrial sequences integrate into the nuclear genome, the most likely fate of these sequences is to mutate as pseudogenes; therefore, the entire sequence, including conserved regions, will evolve at the same rate because of a lack of evolutionary constraints. Therefore, it is advantageous being able to identify the gene order and conserved region using *$tRNA^{VAL}$* of the PCR product amplified by 12SR and 12SL primer pairs to recognize whether the sequence is our target.

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萬用引子用以增幅脊椎動物完整粒線體 *12S rRNA* 基因

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脊椎動物粒線體 *tRNA^{PHE}* 和 *16S rRNA* 之保守區經比對後設計成引子 12SR 和 12SL 。這些萬用引子可以廣泛地使用於脊椎動物各個主群的動物種，而能利用聚合酶鏈反應來增幅約 1.3 kb 的去氧核醣核酸片段。此產物的長度差異在各群脊椎動物間很小。進一步的序列分析顯示此片段含有完整的 *12S rRNA* 和 *tRNA^{PHE}* 及約 200 kb 的 *16S rRNA* 。研究分類群後發現上述的引子能用以增幅完整粒線體 *12S rRNA* 。在 12S rRNA 基因被廣泛地用於不同層級類緣分析的同時，這些引子組更能被用在脊椎動物較高階的類緣關係。

關鍵詞：12SL 和 12SR ，引子，聚合酶鏈反應增幅作用，脊椎動物。

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