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Molecular Techniques to Identify Freshwater Eels: RFLP Analyses of PCR-amplified DNA Fragments and Allele-specific PCR from Mitochondrial DNA

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Yeong-Shin Lin, Yu-Ping Poh, Si-Min Lin and Chyng-Shyan Tzeng (2002) Molecular techniques to identify freshwater eels: RFLP analyses of PCR-amplified DNA fragments and allele-specific PCR from mitochondrial DNA. *Zoological Studies* **41**(4): 421-430. Because of morphological ambiguity, traditional classification of freshwater eel elvers (of the genus *Anguilla*) has always been difficult and unreliable. This study analyzes 2 mitochondrial genes viz., cytochrome *b* and 12S ribosomal RNA (rRNA) genes from previous studies, to establish molecular standards for eel identification. Prediction of restriction fragment length polymorphism (RFLP) indicated that a combination of 3 enzyme results for cytochrome *b* revealed good resolution power. We also selected 39 specific nucleotide sites of the 2 genes at which the site is unique for individual species: thus these can potentially be used for identification by polymerase chain reaction (PCR). To verify the accuracy of our predictions, we examined 58 specimens from 4 species by *Dpn*II and *Hae*III digestions and by PCR amplification using species-specific primers. Consistent with the putative mutation rates, more than 95% and 99% of the specimens could be successfully identified by the RFLP and PCR methods, respectively. The molecular techniques developed here can be helpful in eel aquaculture and ecological studies.
http://www.sinica.edu.tw/zool/ zoolstud/41.4/421.pdf

Key words: PCR, RFLP, Identification, *Anguilla*, Elver.

All freshwater eels belong to the genus *Anguilla* (family Anguillidae), and are elopomorph fishes diagnosed by a unique leptocephalus larval stage. Fifteen species and 2 subspecies of *Anguilla* are currently recognized (Ege 1939, Castle and Williamson 1974, Jellyman 1987). However, species identification based on the external morphology of elvers remains difficult.

In East Asia, eel culture is a highly profitable aquaculture industry. However, due to an insufficient understanding of the life cycle, eel fry needed for culturing are only available through the capture of ascending elvers. Occasionally some sellers mix elvers of less-desirable species with *A. japonica*, the most suitable one for aquaculture. A similar situation occurs in Taiwan with the protected species, *A. marmorata*, due to its putative medicinal properties. Most of the variegated specimens in the market are *A. reinhardti* which are imported from Australia. However, some fishermen continue to collect *A. marmorata* from the wild illegally and sell them to restaurants. Efficient identification of eel species is critical for aquaculture management as well as for eel conservation.

Molecular approaches have previously been used to identify *A. anguilla* and *A. rostrata*, two species whose spawning areas partially overlap (Comparini and Rodino 1980, Williams et al. 1984, Avise et al. 1986 1990, Tagliavini et al. 1995, Lehmann et al. 2000). DNA sequence data have also been employed for other *Anguilla* species as well (Aoyama et al. 1996 1999, Tsukamoto and

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Aoyama 1998, Bastrop et al. 2000). The number of examined species in each of these studies was limited, and undistinguishable species pairs were discovered: one between available sequence data of *A. bengalensis labiata* and *A. marmorata* (Ishikawa et al. 1999), and the other between *A. mossambica* and *A. reinhardti* (Bastrop et al. 2000).

To resolve these problems, our previous study used complete sequences of 2 mitochondrial genes viz., cytochrome *b* and 12S ribosomal RNA (rRNA) genes, to identify 12 *Anguilla* species (Lin

Table 1. Samples analyzed

	Cytochrome *b*		12S rRNA
Species	Lin et al. (2001)	Aoyama et al. (2001)	Lin et al. (2001)
Anguilla japonica	AF006702	AF021772	AF266482
	AF006703		AF266483
A. marmorata	AF006704	AF021778	AF266484
	AF006705		AF266485
	AF074863		
A. reinhardti	AF006706	AF021768	AF266486
	AF006707		AF266487
A. bicolor pacific	AF006708	AF021774	AF266488
	AF006709		AF266489
A. bicolor bicolor	AF006710	AF021780[c]	AF266490
A. dieffenbachi	AF006711	AF021770	AF266491
A. australis[a]	AF006712	AF021769	AF266492
	AF006713	AF021775	AF266493
A. anguilla	AF006714	AF021776	AF266494
	AF006715		AF266495
A. rostrata	AF006716	AF021767	AF266496
	AF006717		AF266497
A. malgumora[b]	AF006718[d]	AF021779[d]	AF266498
	AF006719[d]		AF266499
A. mossambica	AF074864	AF021782	AF266500
	AF074865		AF266501
A. bengalensis labiata[b]	AF074866		AF266502
A. bengalensis bengalensis[b]		AF021783	
A. megastoma		AF021771	
A. interioris		AF021773	
A. celebesensis		AF021777	
A. obscura		AF021781	

[a]The 2 subspecies of *A. australis* were considered to be the same species by Jellyman (1987).
[b]*A. borneensis* and *A. nebulosa* are synonyms of *A. malgumora* and *A. bengalensis*, respectively.
[c]The sequence of *A. bicolor bicolor* by Aoyama et al. (2001) is incorrect as described in table 2.
[d]Specimens of *A. malgumora* obtained in the 2 studies might be different species and are treated separately in this study.

Table 2. Nucleotide substitution matrix of the cytochrome *b* gene among sequences of the 2 subspecies of *Anguilla bicolor* and *A. mossambica*

	Number of nucleotide differences of the cytochrome *b* gene															
Sample	First 457 base pairs								Last 683 base pairs							
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
[1] *A. bicolor pacifica* 1 (Lin)[a]	-	0	1	5	33	33	33	33	-	1	3	22	28	70	67	71
[2] *A. bicolor pacifica* 2 (Lin)[a]		-	1	5	33	33	33	33		-	2	21	27	69	66	70
[3] *A. bicolor pacifica* (Aoyama)[b]			-	4	33	33	33	33			-	19	25	68	65	69
[4] *A. bicolor bicolor* (Lin)[a]				-	37	37	37	37				-	12	60	57	61
[5] *A. bicolor bicolor* (Aoyama)[b]					-	0	1	0					-	59	56	60
[6] *A. mossambica* 1 (Lin)[a]						-	1	0						-	3	5
[7] *A. mossambica* 2 (Lin)[a]							-	1							-	6
[8] *A. mossambica* (Aoyama)[b]								-								-

[a]From Lin et al. (2001). [b]From Aoyama et al. (2001).

et al. 2001). The phylogeny of freshwater eels thus constructed reveals that (1) *Anguilla* first radiated about 20 million years ago, (2) the ancestors of Atlantic eels did not migrate by drifting through the Tethys Seaway at the leptocephali stage but instead trekked across the Central American isthmus to the Sargasso Sea for spawning at the adult stage, and (3) multiple radiation events occurred at the Metropolis during *Anguilla* evolution. Later, Aoyama et al. (2001) described the same approach using cytochrome *b* and 16S rRNA genes from all 17 species and declared conflicting results. However, the cytochrome *b* sequence of *A. bicolor bicolor* in their study was incorrect. Lin et al. (2000) discussed the arguments and favored the Central American isthmus hypothesis.

Although developments in molecular biology have improved our ability to resolve many problems, identification by DNA sequencing, however, is both costly and time consuming. Restriction fragment length polymorphism (RFLP) analysis, instead, is more economical than DNA sequencing analysis and more reliable than morphology-based judgments. Thus, we included published sequence data, analyzed restriction fragment length polymorphisms, and designed specific primers to amplify the fragments of particular species, with the goal of developing useful molecular techniques for eel identification.

MATERIALS AND METHODS

Table 1 illustrates the sequences used in this study. The substitution numbers among cytochrome *b* sequences of the 2 subspecies of *Anguilla bicolor* and *A. mossambica* displayed in table 2 reveal that the first 457 base pairs (bp) of the sequence of *A. b. bicolor* obtained by Aoyama et al. (2001) are incorrect due to mixing with the sequence of *A. mossambica*. On the other hand, the similarity between cytochrome *b* genes of *A. malgumora* from the 2 different studies is less than 93% (data not shown) and implies that they might belong to different species. Consequently, *A. b. bicolor* and *A. malgumora* from the 2 previous studies were treated separately in this study. Biology WorkBench version 3.2 (http://workbench.sdsc.edu) lists all possible restriction enzymes and cutting sites of the obtained sequences. To select appropriate enzymes for identification, we retained enzymes with sufficient polymorphism among species and eliminated those that vary within a species. Fragments of similar size were undistinguishable on the gel, and RFLP types with such fragments were considered to be the same polymorphism type for eel identification. We also screened sequences to determine specific sites where a unique transversional substitution occurs between 1 species and all others. The variation site can then be designated as the 3' end nucleotide of the specific primer for PCR amplification in order to identify specific species.

Mitochondrial genes can be PCR-amplified and then digested by selected restriction enzymes to demonstrate that the above RFLP analysis can actually be performed. In this study, two enzymes used by Lin (1998), *Dpn*II and *Hae*III, and 58 specimens from the 3 most-common species used for aquaculture, *A. japonica*, *A. anguilla*, and *A. rostrata*, as well as a protected species in Taiwan, *A. marmorata*, were applied to examine intraspecific polymorphism. Specimens were randomly selected from different times, locations, and stages of maturity. Identification of ambiguous specimens by sequencing followed Lin et al. (2001) (data not shown). Crude DNA preparation, primers (L15239 and H16468), and thermal conditions used here to amplify a 1230-bp fragment containing the cytochrome *b* gene also followed Lin et al. (2001). Five microliters of the amplified 1230-bp products were incubated with 1 μl of 10× NEBuffer 3, 0.5 units of *Dpn*II (BioLabs), and water in a total volume of 10 μl at 37°C for 8 h. Similar steps were performed for *Hae*III with 10× NEBuffer 2 (BioLabs) at the same time. Digestion patterns were analyzed by electrophoresis on 2% agarose gels.

We designed 2 species-specific primers according to the specific sites listed in tables 5 and 6 for *A. japonica* and *A. marmorata*, viz., L15996 and H01110, respectively. Intraspecific polymorphism was also searched for among the 58 specimens of the 4 species. The thermal conditions for PCR amplifications and the primers applied (one was specific, and the other was universal for all species) were modified as follows. The first specific reaction for *A. japonica* consisted of 93°C for 3 min, 93°C for 30 sec, 60°C for 40 sec, 72°C for 1 min (repeated 40 times), and 72°C for 10 min, with specific primer L15996 (5'-CAATACTTGCCCTATTCTC-3' 16014) and universal primer H00360 (5'-ATATAATGTTCCTGAAATAGGAACC-3' 00336). The other one specific for *A. marmorata* consisted of 93°C for 3 min, 93°C for 30 sec, 72°C for 40 sec (repeated 40 times), and 72°C for 10 min, with universal primer L00917 (5'-AAAGCACGGCACTGAAGATGC-3' 00937) and specific primer H01110

(5'-GCAAGGCGTTTTGGGCTACT-3' 01091). The primer names indicate the locations in the light or heavy strand, and corresponding positions in the *Crossostoma lacustre* mitochondrial genome (Tzeng et al. 1992). Results of amplifications were also examined by electrophoresis on 2% agarose gels.

RESULTS

The predicted fragments of cytochrome *b* and 12S rRNA genes digested by 8 and 12 restriction enzymes for 18 (containing 2 *A. b. bicolor* and 2 *A. malgumora*, with the 2 subspecies of *A. bengalensis* combined due to be undistinguishable in this

Table 3. Restriction fragment length polymorphism from a 1230-bp fragment containing the cytochrome *b* gene. Fragments of small size were eliminated

	*Dpn*II **		*Hae*II ***		*Hae*III ***		*Taq*I ***	
Species	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type
Anguilla japonica	345, 553	A	614, 616	A	298, 932	A	1230	A
A. marmorata	345, 787	B	1230	B	139, 1091	B	1230	A
A. reinhardti	176, 234, 345, 377	C	614, 616	A	139, 1091	B	263, 967	B
A. bicolor pacifica	234, 256, 297, 345	D	614, 616	A	139, 1091	B	1230	A
A. bicolor bicolor	234, 256, 297, 345	D	614, 616	A	139, 1091	B	249, 981	B
A. dieffenbachi	143, 345, 553	E	1230	B	139, 1091	B	1230	A
A. australis	234, 345, 553	F	1230	B	139, 1091	B	1230	A
A. anguilla	234, 345, 553	F	1230	B	139, 298, 315, 478	C	1230	A
A. rostrata	143, 345, 553	E	1230	B	139, 298, 315, 478	C	548, 682	C
A. malgumora	1132	G	1230	B	298, 932	A	1230	A
A. mossambica	234, 345, 553	F	467, 763	C	139, 1091	B	1230	A
A. bengalensis	345, 696	H	467, 616	D	139, 1091	B	1230	A
A. megastoma[a]	234, 345, 553	F	147, 467, 616	E	139, 1091	B	1230	A
A. interioris[a]	345, 787	B	614, 616	A	1230	D	249, 981	B
A. celebesensis[a]	143, 176, 345, 377	I	1230	B	139, 1091	B	567, 663	C
A. obscura[a]	234, 256, 297, 345	D	614, 616	A	1230	D	1230	A
A. malgumora[a]	234, 345, 553	F	467, 763	C	139, 478, 613	E	1230	A
A. bicolor bicolor[a]	143, 345, 553	E	147, 467, 616	E	139, 1091	B	1230	A

	*Hinc*II **		*Apo*I *		*Sau*96I **		*Sty*I ***	
Species	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type
Anguilla japonica	549, 601	A	214, 737	A	296, 934	A	147, 444, 639	A
A. marmorata	549, 601	A	162, 395, 541	B	1230	B	147, 444, 639	A
A. reinhardti	549, 681	B	1098	C	1230	B	147, 444, 639	A
A. bicolor pacifica	258, 291, 601	C	214, 884	D	1230	B	147, 444, 639	A
A. bicolor bicolor	258, 291, 601	C	214, 884	D	1230	B	147, 444, 639	A
A. dieffenbachi	258, 291, 681	D	214, 884	D	1230	B	147, 444, 639	A
A. australis	258, 291, 681	D	1098	C	1230	B	147, 444, 639	A
A. anguilla	258, 291, 681	D	214, 884	D	296, 315, 619	C	1230	B
A. rostrata	258, 291, 681	D	214, 884	D	296, 315, 619	C	1230	B
A. malgumora	258, 892	E	214, 344, 540	E	296, 934	A	147, 444, 639	A
A. mossambica	258, 291, 681	D	214, 344, 540	E	1230	B	147, 1083	C
A. bengalensis	549, 601	A	214, 884	D	1230	B	147, 444, 639	A
A. megastoma[a]	549, 601	A	1098	C	1230	B	147, 1083	C
A. interioris[a]	258, 892	E	1098	C	1230	B	147, 444, 639	A
A. celebesensis[a]	549, 601	A	1098	C	296, 934	A	147, 1083	C
A. obscura[a]	549, 681	B	214, 884	D	1230	B	147, 444, 639	A
A. malgumora[a]	258, 291, 681	D	214, 884	D	611, 619	D	444, 786	D
A. bicolor bicolor[a]	258, 291, 601	C	214, 884	D	1230	B	147, 444, 639	A

[a]Patterns are predicted according to Aoyama et al. (2001).
The number of asterisks indicates the economic rank of the enzyme; more asterisks indicate that the enzyme is more economical.

study) and 12 species, respectively, are listed in tables 3 and 4. Fragments of small size (less than 90 bp) or of larger size but shared by each species (about 90~150 bp) were eliminated to simplify the tables. The *Dpn*II digestion pattern of *A. dieffenbachi* predicted from Aoyama et al.'s (2001) sequence data is "F" instead of "E". Four more *A. dieffenbachi* specimens were experimentally examined as the "E" type as predicted from Lin et al.'s (2001) study (data not shown). This result implies that the "F" type *Dpn*II digestion pattern of *A. dieffenbachi* should be intraspecific polymorphism. In our analysis, no single restriction enzyme was able to completely separate all freshwater eel species, but combinations of different enzyme digestion results could aid in species identification. Considering the cytochrome *b* gene first, a *Dpn*II + *Hae*II + *Hae*III pair was able to recognize all species except the 2 subspecies of *A. bicolor*. To distinguish these 2 subspecies, it was necessary to add *Taq*I. Note that a specimen with variations at cutting sites could lead to misidentification. Treating samples with 6 enzyme combinations (*Dpn*II + *Hae*II + *Hae*III + *Taq*I + *Hinc*II + *Apo*I) will provide at least 2 specific polymorphism types between species (only 1 for the 2 subspecies of *A. bicolor*) and can remind users to double check whenever variations occur. Combining 4 enzymes (*Rsa*I, *Tsp*509I, *Hpa*II, and 1 of the 3 enzymes, *Ava*II, *Eco*O1091, and *Sau*96I) is necessary to distinguish the 12 available species, if the 12S rRNA gene is considered.

We recognized 39 specific nucleotide sites of cytochrome *b* and 12S rRNA genes that can distinguish one species from all others as indicated in tables 5 and 6. Transversional substitutions occur with more difficulty than do transitional substitutions and are more easily distinguished by PCR amplification reactions. Only 3 transitional substitutions are represented for they can be designated at the same primer with other neighboring transversional substitutions. All species, except for *A. b. bicolor* and *A. interioris*, have at least 1 such specific site. Designating the presented site as the 3' end nucleotide of the specific primer allows the recognition of a particular species by PCR amplifi-

Table 4. Restriction fragment length polymorphism from an entire 12S rRNA gene. Fragments of small size were eliminated. Similar-sized fragments were considered as the same polymorphism type for eel identification

Species	*Rsa*I **		*Tsp*509I **		*Hpa*II ***		*Hha*I ***		*Hin*P1I ***		*Nla*III *	
	Fragments (bp)	Type	Fragments (bp)	Type	Fragment (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type
Anguilla japonica	94, 774	A	106, 163, 681	A	577	A	123, 764	A	125, 764	A	197, 702	A
A. marmorata	94, 102, 673	B	106, 164, 681	A	375	B	123, 828	B	125, 826	B	197, 703	A
A. rainhardti	768	C	270, 682	B	465	C	123, 829	B	125, 827	B	197, 704	A
A. bicolor pacifica	94, 767	A	106, 164, 681	A	465	C	123, 828	B	125, 826	B	197, 703	A
A. bicolor bicolor	94, 767	A	270, 681	B	577	A	123, 828	B	125, 826	B	197, 703	A
A. dieffenbachi	94, 766	A	106, 163, 600	C	465	C	950	C	950	C	197, 284, 418	B
A. australis	94, 767	A	106, 164, 600	C	465	C	123, 828	B	125, 826	B	142, 197, 561	C
A. anguilla	94, 102, 674	B	106, 163, 602	C	579	A	123, 766	A	125, 766	A	197, 704	A
A. rostrata	94, 774	A	106, 163, 600	C	577	A	123, 764	A	125, 764	A	197, 702	A
A. malgumora	94, 102, 666	B	106, 164, 682	A	376	B	123, 829	B	125, 827	B	197, 704	A
A. mossambica	95, 776	A	107, 116, 125, 164,	D	701	D	118, 835	B	116, 837	B	164, 704	D
A. bengalensis	94, 767	A	270, 681	B	465	C	123, 828	B	125, 826	B	197, 703	A

Species	*Ava*II ***		*Eco*O1091 ***		*Sau*96I **		*Taq*I ***		*Apo*I *		*Eco*RI ****	
	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type	Fragments (bp)	Type
Anguilla japonica	939	A	939	A	939	A	171, 681	A	163, 787	A	163, 787	A
A. marmorata	940	A	940	A	940	A	171, 682	A	164, 787	A	164, 787	A
A. rainhardti	941	A	941	A	941	A	171, 683	A	952	B	952	B
A. bicolor pacifica	290, 650	B	290, 650	B	290, 650	B	171, 682	A	164, 787	A	164, 787	A
A. bicolor bicolor	290, 650	B	290, 650	B	290, 650	B	171, 682	A	951	B	951	B
A. dieffenbachi	939	A	939	A	939	A	171, 202, 479	B	163, 787	A	163, 787	A
A. australis	290, 650	B	290, 650	B	290, 650	B	171, 203, 479	B	164, 787	A	164, 787	A
A. anguilla	941	A	941	A	941	A	171, 202, 481	B	163, 789	A	163, 789	A
A. rostrata	939	A	939	A	939	A	171, 202, 479	B	163. 787	A	163. 787	A
A. malgumora	290, 651	B	290, 651	B	290, 651	B	171, 683	A	164, 788	A	164, 788	A
A. mossambica	942	A	942	A	942	A	171, 203, 480	B	164, 232, 557	C	164, 232, 557	C
A. bengalensis	940	A	940	A	940	A	171, 682	A	951	B	951	B

The number of asterisks indicates the economic rank of the enzyme; more asterisks indicate that the enzyme is more economical.

cation analysis.

Fifty-eight available samples from 4 species (*A. anguilla*, *A. japonica*, *A. marmorata*, and *A. rostrata*) were examined by RFLP with *Dpn*II and *Hae*III separately as well as by the PCR method with primers L15996/H00360 and L00917/H01110, to check the feasibility and accuracy of these techniques (Table 7). Intraspecific polymorphism introduced a total of 5 unexpected digestion patterns from 116 RFLP tests (error ratio, 4.31%) and 1 false-positive signal in the PCR amplification analysis (error ratio, 0.86%).

DISCUSSION

We can estimate the possibility (*P*) of the intra-species polymorphism of RFLP analysis from Lin et al.'s (2001) results,

$$P = (\gamma / N) \times [(N / 4^n) \times n] = \gamma \times n / 4^n, \quad (1)$$

where γ is the average intraspecific diversity, *N* is the nucleotide length of the gene, and *n* is the number of recognition sequence sites of the restriction enzyme. The average intraspecific substitution of cytochrome *b* gene in Lin et al.'s (2001) study was 3.625 bp, and was 4.793 bp when including Aoyama et al.'s (2001) data. Both *Dpn*II and *Hae*III are 4-bp recognition enzymes. *P* was thus calculated to be 0.0566 and 0.0749, which are close to that of our polymorphism analyses (observed intraspecific polymorphism (*O*) = 5/116 = 0.0431) in this study, indicating that we may have misidentified approximately 1 specimen per 20 examined samples. Among 63 leptocephali analyzed by Aoyama et al. (1999), 44 were identified morphologically, and of these, 35 (79.5%) agreed with the identification by sequencing their

Table 5. Specific nucleotide sites of the cytochrome *b* gene. Position numbers are designated according to the *Crossostoma lacustre* mitochondrial genome (Tzeng et al. 1992)

Species	1111111111111111111111111111111111111111 5555555555555555555556666666666666666 3334445566777488899900011112222222 3 9990691102335658934911614670223355 4 2580361768362336243848941577025668 2
Anguilla japonica	TCATCTCAACTATTTAAATACACCCCCACCGCATA
A. marmorata	TCAACTACACCCTCTAAAAAAACTTTCACTGCACA
A. reinhardti	TCATCTGCACTCTCTAAAAAAACACCCGCTGCACA
A. b. pacifica	TCATCTACACCTTCTAAAATAACTTCAACTGCATA
A. b. bicolor	TCATCTACACCCTCTAAAAAAATCTCAACTGCATA
A. dieffenbachi	TCATCTACATCCTCTAAAAAAACCTACACTGCATA
A. australis	TCACCCATATCCTCTTTAAAAACCCCCACCGCGAA
A. anguilla	TTATCCACTTCCTCCAAAAAAGTCCCTACTGCATA
A. rostrata	TTATCCACACCTTCCAAAAAAATTCCCTACTGCATA
A. malgumora	TCATCTACACCCTCTAACAAAACTTCCACTGCATA
A. mossambica	TCATCTATATTCTCAAAAAAAAACACCACAATATA
A. bengalensis	TCATCTACACACTCTAAAAAAACTTCCACTGCATA
A. megastoma[a]	TCATATATATCCTATAAAAAAACCTTCACTGCACA
A. interioris[a]	TCATCTATACCCTCTAAAAAAACTTCCTCTGCACA
A. celebesensis[a]	TCATCCGTACCCTCTAAAAAAACCTCCAATGCACC
A. obscura[a]	TCATTAACACCCTCTAAAAAAACTCCCTCTGCATA
A. malgumora[a]	AATTCCACAACCACCAAAAAAATTCCCACCGCATA
A. b. bicolor[a]	TCATCTATATTCTCTAAAAAAATCTCAACTGCATA

[a]Patterns are predicted according to Aoyama et al. (2001).

The 32 highlighted sites indicate unique transversional substitutions (containing 1 for the 2 subspecies of *Anguilla bicolor* and the other for *A. interioris* and *A. obscura*).

The underlined sites are neighbors and can be designed at the same primer, although 3 of them only have unique transitional substitutions with other species.

16S rRNA genes. This result implies that the accuracy of RFLP analysis is much better and more reliable than that of morphological identification.

The possibility of intraspecific polymorphism of PCR amplification with one specific primer can be estimated as follows:

$$P = \gamma / N. \quad (2)$$

With N equals to 1140 for the cytochrome *b* gene, P is thus reduced to 0.0032 and 0.0042. Even when we consider that most of the substitutions occur at the 3rd codon, and given that the last nucleotide of the specific primer is also at the 3rd codon, P is calculated to be 0.0095 and 0.0126. That implies that there is only 1 misidentified specimen per 100 examined samples. The P value for the 12S rRNA gene is 0.0022, and is 0.0044 when assuming that all substitutions occur within the loop regions. Our experimental results also support this number (O = 1/116 = 0.0086). Obviously, appropriate primers with more-specific nucleotides can improve the identification specificity.

Previous experiments have proven that 1 transversional substitution is able to distinguish 1 species from all others. However, the efficiency is not significant until the annealing temperature was set to 72°C for the specific primer of *A. marmorata*. Two specific primers could increase the efficiency, although the accuracy would slightly decrease. Nevertheless, to resolve the deficiency of perceptible sites for *A. b. bicolor* and *A. interioris*, combining 2 specific primers can differentiate the 2 subspecies of *A. bicolor*, and between *A. interioris* and *A. obscura*, for example, with 1 primer specifically for *A. b. pacific* (3' end nucleotide at 15998) and the other for both *A. bicolor* species, respectively (3' end nucleotide at 16177), or with 1 primer specifically for *A. obscura* (3' end nucleotide at 15496) and the other for both *A. interioris* and *A. obscura* (3' end nucleotide at 16207).

In this study, the 2 subspecies of *A. bengalensis* could not be distinguished by RFLP or PCR amplification using the cytochrome *b* gene, because the substitution number between them is 7, even less than some of the intraspecific diversity of *A. anguilla* (AF006715 with AF006714 or AF021776) and *A. marmorata* (AF006704 with AF021778 or AF074863). However, only 1 specimen for each subspecies is not convincing to suggest converging the 2 subspecies. A large-scale examination of *A. bengalensis* is necessary to resolve this issue. Noticeably, specimens of *A. malgumora* obtained by Lin et al. (2001) and Aoyama et al. (2001) showed significant differences in RFLP types, PCR amplification analyses and sequences. Bauchot et al. (1993) transferred *A. borneensis* in Ege (1939) as a synonym of *A. malgumora*. The *A. malgumora* specimens of Lin et al. (2001) consist of 10 glass eels from the Philippines provided by Dr. W. N. Tzeng. The

Table 6. Specific nucleotide sites of the 12S rRNA gene

Species	0 1 0 - 0 8 7	0 1 0 - 0 9 1	0 1 2 - 2 4 5	0 1 2 - 2 4 8	0 1 3 - 3 1 7	0 1 3 - 3 2 4	0 1 8 - 8 5 7	0 1 8 - 8 6 1
Anguilla japonica	CCCGT		TTACA		GGATA		CAAA	
A. marmorata	CTCAAA		TTACA		GGACA		CAAA	
A. reinhardti	CCCAC		TTTCAT		GGATA		CAAA	
A. b. pacifica	CTAAAC		TTACA		GGACA		CAAA	
A. b. bicolor	CTAAAC		TTACA		GGACA		CAAA	
A. dieffenbachi	CTTAC		TTACA		GGACA		CAAG	
A. australis	CTTTAC		TTACA		GGACA		CAAG	
A. anguilla	CCAAC		TTACA		GGAACAT		CAAA	
A. rostrata	CCAAT		TTACA		GGACA		CAAG	
A. malgumora	CCAAAT		TTACA		GGAACA		CAAA	
A. mossambica	CTTCAC		TTACA		TGATA		CAAA	
A. b. labiata	CCAAAT		TTACA		GGATA		AATA	

Position numbers are designated according to the *Crossostoma lacustre* mitochondrial genome (Tzeng et al. 1992).

specimens were identified with Ege's (1939) key (number of vertebrae and AD/TL) as described in Lin (1998), and all of them were confirmed to be the same species by sequencing. Aoyama et al. (2001) collected 1 *A. malgumora* specimen from Borneo and measured the morphological features without the number of vertebrae. According to Lin et al. (2000), these 2 samples should be different species with similar morphological characters. To confirm this assumption, more specimens from these 2 sample locations and the type specimens of *A. malgumora* and *A. borneensis* should be included for further study; our RFLP and specific PCR amplification analyses could serve as simple but powerful tools for screening.

The molecular techniques applied in this study have previously been utilized for salmon (Russell et al. 2000), perch (Asensio et al. 2000), and sardine (Sebastio et al. 2001) species identification in the food industry. Compared with their results, our RFLP patterns can recognize 16 species, much more than theirs, with only 3 restriction enzymes. Applications of species-specific amplification to identify fish species are few, however, this technique is frequently applied for microbiology studies (e.g., Zhan et al. 2001). Comparing 3 recently examined mitochondrial genes, Lin et al. (2000) illustrated that the cytochrome *b* gene is more divergent than are the 12S and 16S rRNA genes, and is more appropriate for constructing freshwater eel identification systems as indicated in this study. Accurate sequences are necessary to construct a reliable identification system. The cytochrome *b* sequence of *A. b. bicolor* from Aoyama et al. (2001) was mixed with that of *A. mossambica*, and this generated incorrect RFLP patterns in table 3. Such circumstances might cause some serious problems and should be avoided.

As to economic aspects, one PCR reaction costs less than US$ 1, while 1 unit of the restriction enzyme for RFLP analysis requires an extra US$ 0.01-1.00. Compared with these techniques, sequencing reactions perfectly recognize species, but the price for 1 sequencing reaction is about US$ 15, much more expensive than the previous 2 methods. RFLP analysis has low costs and moderately high accuracy (95%). As compared to sequencing and RFLP, PCR amplification is the cheapest, easiest to handle, and is a good alternative if appropriate primers can be designed (accuracy = 99%). Taking accuracy and cost into consideration, the molecular methods established here have strong advantages over conventional methods of eel identification, and thus can benefit the eel aquaculture industry and facilitate research to understand the life cycle of freshwater eels.

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Table 7. Intraspecific polymorphism analyzed by RFLP and specific PCR amplification

Species	No. of sample	Expected/unexpected RFLP type numbers		Positive amplification numbers for PCR with a specific primer	
		*Dpn*II	*Hae*III	L15996/H00360	L00917/H01110
Anguilla japonica	15	15 / 0	14 / 1	15	0
A. marmorata	15	14 / 1	15 / 0	0	15
A. anguilla	10	10 / 0	8 / 2	0	0
A. rostrata	18	17 / 1	18 / 0	1	0

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淡水鰻魚的分生鑑定技術：以粒線體去氧核醣核酸進行限制酵素片段長度多型性分析以及專一引子的聚合酵素連鎖反應分析

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由於淡水鰻魚的鰻苗在外型上十分相似而難以區別，傳統的分類工作面臨了許多困難和不確定性。本篇研究參考已經發表的兩個粒線體基因（cytochrome *b* 及12S rRNA）建立了鰻魚鑑定的分子標準。根據推測得到的限制酵素片段長度多型性，結合三個限制酵素就能在cytochrome *b* 基因有很好的鑑別力。另外我們也在這兩個基因上共找到39個特別的核苷酸位址，可用來設計專一的引子進行聚合酵素連鎖反應分析。為了檢驗這些預測的正確性，我們選取四種共58個樣本進行*Dpn*II和*Hae*III的限制酵素片段長度多型性分析以及專一引子的聚合酵素連鎖反應分析。結果顯示，他們分別都有超過95%和99%的正確率，和根據突變率預測的結果相符合。因此，本篇研究所提出來的分生鑑定技術，對鰻魚養殖業以及生態研究工作應有一定的參考價值。

關鍵詞：聚合酵素連鎖反應，限制酵素片段長度多型性，鑑定，鰻鱺，鰻苗。

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