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MtDNA diversity of the critically endangered Mekong giant catfish (*Pangasianodon gigas* Chevey, 1913) and closely related species: implications for conservation

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Abstract

Catfishes of the family Pangasiidae are an important group that contributes significantly to the fisheries of the Mekong River basin. In recent times the populations of several catfish species have declined, thought to be due to overfishing and habitat changes brought about by anthropogenic influences. The Mekong giant catfish *Pangasianodon gigas* Chevey, 1913 is listed as Critically Endangered on the IUCN Red List. In the present study, we assessed the level of genetic diversity of nine catfish species using sequences of the large subunit of mitochondrial DNA (16S rRNA). Approximately 570 base pairs (bp) were sequenced from 672 individuals of nine species. In all species studied, haplotype diversity and nucleotide diversity ranged from 0.118 ± 0.101 to 0.667 ± 0.141 and from 0.0002 ± 0.0003 to 0.0016 ± 0.0013, respectively. Four haplotypes were detected among 16 samples from natural populations of the critically endangered Mekong giant catfish. The results, in spite of the limited sample size for some species investigated, indicated that the level of genetic variation observed in wild populations of the Mekong giant catfish (haplotype diversity $= 0.350 \pm 0.148$, nucleotide diversity $= 0.0009 \pm 0.0008$) is commensurate with that of some other related species. This finding indicates that (1) wild populations of the Mekong giant catfish might be more robust than currently thought or (2) present wild populations of this species carry a genetic signature of the historically larger population(s). Findings from this study also have important implications for conservation of the Mekong giant catfish, especially in designing and implementing artificial breeding programme for restocking purposes.

Introduction

An understanding of the level of genetic diversity of rare and endangered species can contribute to knowledge of their evolutionary history and potential and is critical to developing strategies for their conservation and management. Genetic diversity influences the adaptive flexibility of a species to environmental changes (Vrijenhoek, 1994) and is an important factor in the conservation of endangered species. Although there are instances where populations survive over long periods of time despite low levels of genetic variations (Groombridge *et al*., 2000; Visscher *et al*., 2001), the long-term risks posed by low levels of genetic variation have made the management and restoration of the latter a major aim in conservation (Frankham, Ballou & Briscoe, 2002).

Pangasianodon gigas Chevey, 1913, the Mekong giant catfish endemic to the Mekong River basin, is one of the largest freshwater fishes of the world, up to 300 kg in weight and 300 cm in length (Hogan *et al*., 2004). This species is considered to be Critically Endangered (IUCN, http://www.iucn.org) and is also listed in Appendix I of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Conservation initiatives by the IUCN have included a 'buy-and-release' scheme to reduce fishing-related mortality (Hogan *et al*., 2004). Mekong giant catfishes are commercially farmed in Thailand and, despite the apparent endangered status of wild stocks, a substantial population of first-generation broodstock is held in captivity.

Several species of the family Pangasiidae are important food fish in the South-east Asian region. These species contribute significantly to regional fisheries, especially the fisheries in the Mekong, which supports one of the most significant riverine fisheries in the world (Coates, 2002).

Some of the pangasiid species such as *Pangasius* (= *Pangasianodon*) *hypophthalmus* (Sauvage 1878) and *Pangasius bocourti* Sauvage 1880 are widely cultured in the lower Mekong River basin (Trong, Nguyen & Griffiths, 2002).

Populations of the Mekong giant catfish and other closely species are reported to have markedly declined over the years (Sverdrup-Jensen, 2002). The decline in wild populations is thought to be due to overfishing and habitat destruction caused by anthropogenic activities (Coates, 2002). The numbers of Mekong giant catfish caught from the wild, in Chiangrai Province, Thailand for example, have declined from a peak of about 65 individuals in 1990 to fewer than five in 1997 (Pholprasith & Tavarutmaneegul, 1997), and in the 2001 and 2002 seasons (April–May) none were caught (Poulsen *et al.*, 2004). This species is also of important cultural value, particularly in Lao PDR and Thailand, and in the upper reaches of the river the annual fishery is preceded by a traditional ceremony. Pangasiid fishes are likely to be at high risk as most fisheries for such species take place during their spawning migrations and species are generally of large size and mature slowly (Warren, Chapman & Singanouvong, 1998; Sverdrup-Jensen, 2002).

Despite the importance and popularity of pangasiid catfishes to Mekong riparian countries, details of their biology, especially levels of genetic diversity, are not well documented (Mattson *et al.*, 2002; Poulsen *et al.*, 2004). The only genetic investigation to date is a study of the phylogenetic relationships among pangasiid catfishes by Pouyaud *et al.* (2000), in which intraspecific genetic diversity was not examined. In most species and populations, the amount of genetic variation and thus the potential threats posed by limited variation are unknown.

In this study we estimated the levels of genetic variation of both wild and captive populations of the Mekong giant catfish, and that of other closely related species using sequences of the large subunit ribosomal RNA (16S rRNA) gene region of the mitochondrial genome. One needs to also appreciate the difficulties of obtaining samples of wild stocks of a highly endangered species such as the Mekong giant catfish, of which only one or two individual fish are caught in a year in the commercial fishery, and of which knowledge on the spawning grounds and life-history stages is almost unknown.

Materials and methods

Sampling

Details of sampling localities and sample sizes are presented in Table 1 and Fig. 1. Finclips from 16 individuals of *Pangasianodon gigas* were collected between 2002 and 2005 from commercial catches in the Mekong River and its tributaries (from Cambodia and Thailand). In addition, finclips of 127 individuals from captive-bred stocks held at four government and three private hatcheries in Thailand were also obtained (Table 1).

Finclips of 95 individuals of the only congener (i.e. species belonging to the same genus) of the giant catfish *Pangasianodon hypophthalmus* were collected. Samples of 435 individuals of seven other species, including five species of the genus *Pangasius*, that is *P. bocourti*, *Pangasius conchophilus* Roberts & Vidthayanon, 1991, *Pangasius larnaudii* Bocourt, 1866, *Pangasius macronema* Bleeker, 1851 and *Pangasius sanitwongsei* Smith, 1931, and one species each of the two other closely related genera, that is *Helicophagus waandersii* Bleeker, 1858 and *Pteropangasius pleurotaenia* Sauvage, 1878, from commercial catches in the Mekong River and some from the Chao Phraya River were also collected (Table 1). All finclips were preserved in 95% ethanol until required.

Laboratory procedures

Genomic DNA was extracted from 20–50 mg of finclip tissue according to the method described by Taggart *et al.* (1992) with slight modifications. DNA was suspended in TE buffer (10 mM Tris-HCl pH 7.5; 1 mM EDTA pH 8.0) and stored at 4 °C until required.

A partial region of mitochondrial 16S rRNA gene was amplified using primers 16Sar (5′-CGC CTG TTT AAC AAA AAC AT-3′) and 16Sbr (5′-CCG GTC TGA ACT CAG ATC ATG T-3′) (Palumbi *et al.*, 1991). Polymerase chain reaction (PCR) was performed in a total volume of 30 μL containing 50 ng μL^{-1} of template DNA, 1 × PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPS, 0.5 μM of each primer and 1 unit of Taq Polymerase (Promega, Madison, WI, USA). Initial denaturation at 94 °C for 3 min was followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

The majority of samples was analysed at the Laboratory of Population Genetic Informatics, Tohoku University, Japan, where PCR products were purified with ExoSAP-IT (usb) and sequenced in an ABI Prism® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The remaining samples were sent to Macrogen Inc., Republic of Korea, for purification and sequencing. All samples were sequenced in both directions to check the validity of the sequence data.

Data analysis

Sequences were viewed and edited using MEGA3.1 (Kumar, Tamura & Nei, 2004) and then aligned using ClustalW as implemented in the same software. Data were then imported into Arlequin version 2.0 (Schneider, Roessli & Excofier, 2000) for further analysis.

Molecular diversity indices within species, that is haplotype diversity (h, the probability that two randomly chosen haplotypes are different) and nucleotide diversity (π, the probability that two randomly chosen homologous nucleotides are different), were estimated (Nei, 1987). Relationships between intraspecific haplotypes within each species were assessed using the molecular-variance parsimony technique (minimum spanning networks) using the same software.

Table 1 Sample codes, sample origins (MK, Mekong River basin; CP, Chao Phraya River basin; CS, captive stock), localities, and sample size for populations of pangasiid species analysed in the present study

Origin	Locality	Year of collection	Sample size
Pangasianodon gigas			
MK	Tonle Sap, Cambodia	2004	1
MK	Chiangrai Province	2004–2005	11
MK	Nakornpanom Province	2001	1
MK	Ubonratchatani Province	2002	3
CS	Inland Fisheries Research Institute, Ayutthaya Province	2004	4
CS	Chiangmai Inland Fisheries Research and Development Centre	2004	31
CS	Maejo University, Chiangmai Province	2003	9
CS	Hatchery, Jaran Farm, Chiangrai Province	2003	11
CS	Hatchery, Wangplabug Farm, Chiangrai Province	2004	39
CS	Phayao Inland Fisheries Research and Development Centre	2004	14
CS	Hatchery, Chaomudcha Farm, Supanburi Province	2004	18
Pangasianodon hypophthalmus			
MK	Tonle Sap, Cambodia	2004	18
MK	Chiangrai Province	2004	4
MK	Nakornpanom Province	2003	12
MK	Nongkhai Inland Fisheries Research and Development Centre	2005	11
CP	Ayutthaya Province	2005	20
CP	Patumtani Province	2004	10
CP	Sakaekrang River, Uthaitani Province	2005	20
Pangasius bocourti			
MK	Chiangrai Province	2004	4
MK	Nongkhai Province	2005	2
MK	Nakornpanom Province	2004	33
MK	Ubonratchatani Province	2005	3
Pangasius conchophilus			
MK	Mukdahan Province	2004	7
MK	Nongkhai Province	2005	13
MK	Nakornpanom Province	2004	6
MK	Sakonnakorn Inland Fisheries Research and Development Centre	2004	11
Pangasius larnaudii			
MK	Mekong River, Cambodia	2004	27
MK	Mekong River, Nongkhai Province	2003	6
MK	Nakornpanom Province	2003	8
MK	Ubonratchatani Province	2004	27
CP	Chainat Province	2003	49
CP	Pichit Province	2003	45
CP	Pisanulok Province	2003	4
CP	Patumtani Province	2004	51
Pangasius macronema			
MK	Nongkhai Province	2005	14
MK	Nakornpanom Province	2004	3
Pangasius sanitwongsei			
MK	Chiangrai Province	2003	1
MK	Nakornpanom Province	2003	44
MK	Ubonratchatani Province	2003	10
MK	Sakonnakorn Inland Fisheries Research and Development Centre	2004	10
Helicophagus waandersii			
MK	Nongkhai Province	2004–2005	21
MK	Nakornpanom Province	2004	24
Pteropangasius pleurotaenia			
MK	Nongkhai Province	2005	3
MK	Nakornpanom Province	2004	2
MK	Ubonratchatani Province	2005	2
MK	Nongkhai Inland Fisheries Research and Development Centre	2005	5

Unless otherwise stated the samples were from different locations in Thailand.

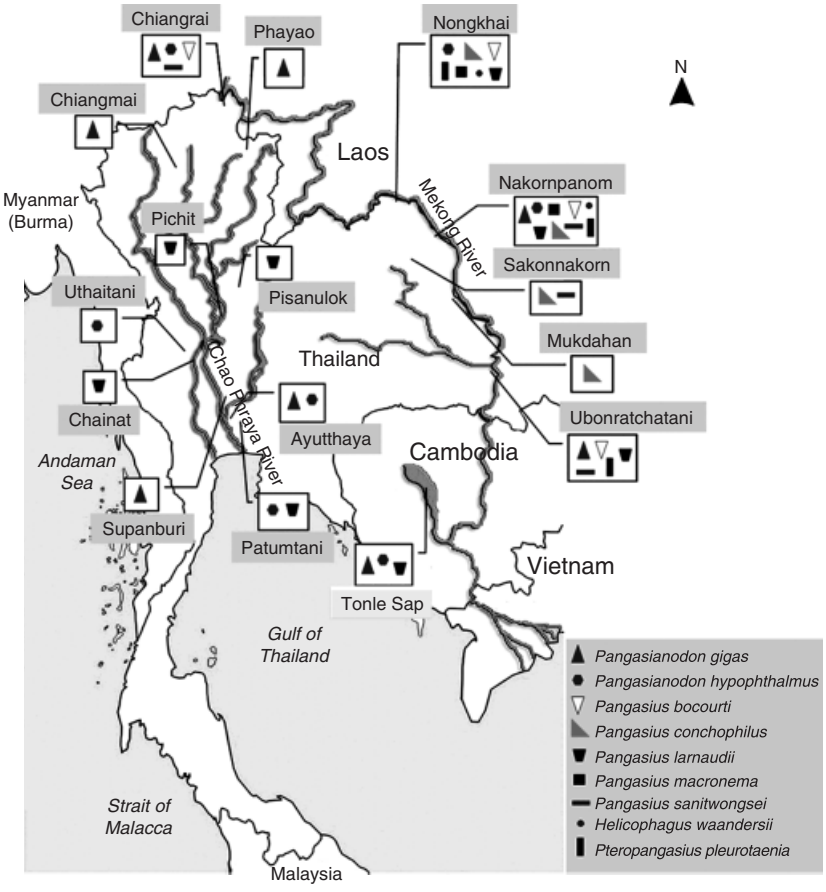

Figure 1 Sampling localities of the nine catfish species in the present study.

Demographic history was investigated by analysing mismatch distributions of pairwise differences between all wild-caught individuals of each species. This kind of analysis is able to discern whether a population/species has undergone rapid expansion (possibly after a bottleneck) or has remained stable over time. It has been demonstrated that population expansion generates a unimodal distribution (similar to a Poisson curve) and stable populations typically produce a multimodal distribution (Slatkin & Hudson, 1991; Rogers & Harpending, 1992). We analysed the shape of the mismatch distribution for each species to test whether the presently observed genetic variation fit an equilibrium model. The interpreted data were subjected to a goodness-of-fit test between the observed and simulated data (Harpending, 1994).

The time of possible population expansions (t, in number of generations) was calculated through the relationship $\tau = 2ut$ (Rogers & Harpending, 1992), where τ is the mode of the mismatch distribution, and u is the mutation rate of the sequence considering that $u = 2\mu k$ (μ is the mutation rate per nucleotide and k is the number of nucleotides). A mutation rate of 1.0% per nucleotide per million years (Myr) was used, as the 16S rRNA gene is considered as one of the most conserved genes in the mtDNA genome (Simon *et al.*, 1994) although it is accepted that the mean rate of evolution of fish mtDNA is 1.0–2.0% (Donalson & Wilson, 1999). As there is no reliable information on maturation age in the wild of any catfish species studied, we used the data that are available in captivity for several species, for example 10–16 years for *Pangasianodon gigas* (Meng-Umphan, 2000), 2–5 years for *Pangasianodon hypophthalmus* (Pimonbud, Udomkarn & Meewan, 1994) and 4–5 years for *P. larnaudii* (Pongsirijan, Rungtongbaisuree & Pongjanyakun, 2001), 6–7 years for *P. sanitwongsei* (Unakornsawad, Tripolaksorn & Yodpaen, 1998), 4–5 years for *P. bocourti* (Pongmaneerat *et al.*, 2006). For species with no information available, we applied the average generation time of 4–5 years.

Arlequin 2.0 (Schneider *et al.*, 2000) was also used to test for departures from mutation-drift equilibrium with Tajima's D test (Tajima, 1989). The statistical significance of this neutrality test was obtained by generating samples in accordance with the hypothesis of selective neutrality and population equilibrium, using a coalescent simulation algorithm as adapted from Hudson (1990).

Statistical testing for population differentiation in each species (where applicable) involved an exact test (Raymond & Rousset, 1995) of a contingency table based on haplotype frequencies and pairwise comparisons of F_{ST} using analysis of molecular variance (Excoffier, Smouse & Quattro, 1992)

Table 2 Distribution of haplotypes observed in the nine pangasiid catfish species originating from the Mekong (MK) and/or the Chao Phraya (CP) River basins

	Origin				Origin	
Haplotype	MK	CP	Species	Haplotype	MK	CP
Pangasianodon gigas						
Pg01	13	No	*Pangasius macronema*	Pm01	16	NS
Pg02	1	No		Pm02	1	NS
Pg03	1	No				
Pg04	1	No	*Pangasius sanitwongsei*	Ps01	52	NS
				Ps02	9	NS
Pangasianodon hypophthalmus						
Ph01	36	42		Ps03	1	NS
Ph02	0	1		Ps04	1	NS
Ph03	0	7		Ps05	1	NS
Ph04	3	0		Ps06	1	NS
Ph05	1	0				
Ph06	2	0	*Helicophagus*	Hw01	37	NS
Ph07	2	0	*waandersii*	Hw02	3	NS
Ph08	1	0		Hw03	1	NS
				Hw04	1	NS
Pangasius bocourti						
Pb01	26	NS		Hw05	1	NS
Pb02	1	NS		Hw06	1	NS
Pb03	4	NS		Hw07	1	NS
Pb04	2	NS				
Pb05	1	NS	*Pteropangasius*	Pp01	7	NS
Pb06	3	NS	*pleurotaenia*	Pp02	1	NS
Pb07	2	NS		Pp03	2	NS
Pb08	1	NS		Pp04	1	NS
Pb09	1	NS		Pp05	1	NS
Pb10	1	NS				
Pangasius conchophilus						
Pc01	25	NS				
Pc02	1	NS				
Pc03	1	NS				
Pangasisu larnaudii						
Pl01	63	139				
Pl02	2	2				
Pl03	1	0				
Pl04	0	1				
Pl05	1	0				
Pl06	0	1				
Pl07	0	1				
Pl08	0	1				
Pl09	0	1				
Pl10	0	3				
Pl11	1	0				

No, does not occur; NS, not sampled.

based on 1000 permutations of the data matrix. Samples were grouped on the basis of their origins, for example Mekong River basin (MK), Chao Phraya River basin (CP) and captive stock (CS).

Genetic relationships among haplotypes were assessed by neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses using PAUP* version 4b10 (Swofford, 2001). The optimal model of nucleotide evolution for NJ and ML analyses was determined by hierarchical likelihood ratio tests using the software Model Test version 3.7 (Posada & Crandall, 1998). The resultant models were used to calculate pairwise sequence distances and to construct the NJ and ML trees. An unweighted MP and ML heuristic search option was used to search for the best tree with starting trees obtained via stepwise addition of taxa, and each search was replicated 10 times. Branch swapping was implemented using the tree-bisection-reconnection (TBR) option. Confidence limits were assessed using bootstrap procedure (Felsenstein, 1985) with 1000 and 500 pseudoreplicates for NJ and MP, and ML, respectively.

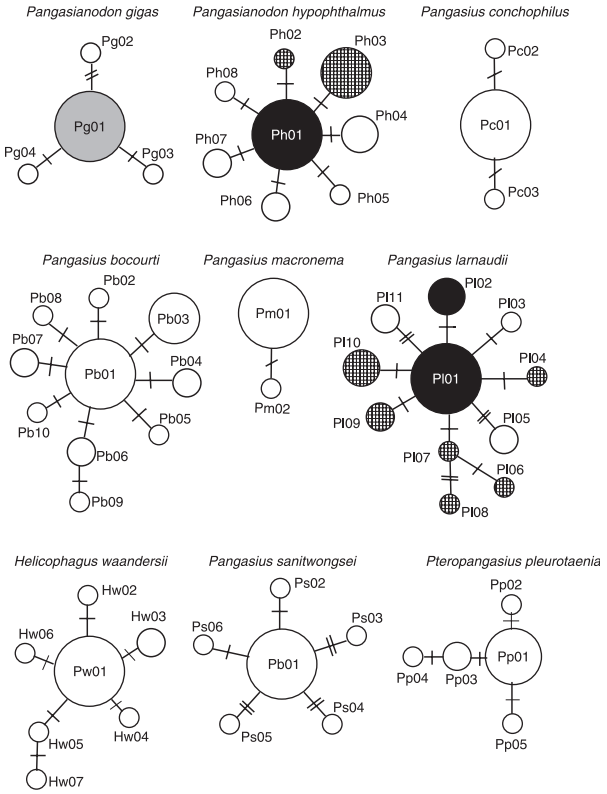

Figure 2 Minimum spanning networks of mtDNA 16S rRNA haplotypes of the nine catfish species studied. Bars across branches indicate single-nucleotide change. The size of each circle, for each species, is an approximate indication of the frequency of the haplotypes present (open circle: found only in the Mekong River system; grey circle: common in captive stocks and in the Mekong; checked shaded circle: found only in Chao Phraya River system; black circle: common in both river systems).

Results

MtDNA 16S rRNA sequence variability and haplotype networks

A total of *c*. 570 bp of the mtDNA 16S rRNA gene region was successfully sequenced for 633 individuals of nine species of pangasiid catfish. Overall, 56 haplotypes were detected for all species, of which the highest number of haplotypes (11) was observed in *P. larnaudi* and the lowest number (two) was detected in *P. macronema*. In the critically endangered Mekong giant catfish, although only 16 individuals were examined, four haplotypes were detected. All individuals ($n = 127$) from captive stock samples of *Pangasianodon gigas* shared one haplotype, which is identical to the most common haplotypes found in the wild samples. Distribution of these haplotypes of each species of different origins (i.e. MK, CP, CS) is presented in Table 2. Sequences of all haplotypes were submitted to GenBank (accession numbers: *Pangasianodon gigas* DQ307046–DQ307049; *Pangasianodon hypophthalmus* DQ334282–DQ334289; *P. bocourti* DQ334290–DQ334299; *P. conchophilus* DQ334300–DQ334302; *P. larnaudii* DQ334303–DQ334313; *P. macronema* DQ334314–DQ334315; *P. sanitwongsei* DQ334316–DQ334321; *H. waandersii* DQ334322–DQ 334328; *Pteropangasius pleurotaenia* DQ334329–DQ334333).

Minimum spanning networks showing relationships among haplotypes within each species are presented in Fig. 2. With the exception of *P. macronema* samples, which consist of only two haplotypes, all the other species showed a star-like phylogeny with one common central haplotype, which is believed to be the most likely ancestral variant according to coalescent theory (Posada & Crandall, 2001). The peripheral mitochondrial variants are connected to the central haplotypes with one to three mutations (Fig. 2).

A summary of mtDNA variation for wild-caught samples of each species is given in Table 3. Overall, all species showed low to moderate haplotype diversity (0.118–0.667) and very low nucleotide diversity (0.0002–0.0016). Of all the species examined, *P. macronema* showed the least genetic variation, while *Pteropangasius pleurotaenia*, even with the smallest sample size, appeared to be the most divergent. *Pangasianodon hypophthalmus*, with much larger sample size, showed similar values of diversity indices to its most closely related and the critically endangered Mekong giant catfish *Pangasianodon gigas*.

Population differentiation

Estimates of genetic differentiation between samples using pairwise F_{ST} and exact tests are given in Table 4. Among three available pairwise tests, significant population differentiation and significant F_{ST} values were observed on only one occasion, for example between the wild and captive stocks of *Pangasianodon gigas* ($F_{ST} = 0.376$, $P = 0.000$, exact test P value = 0.002). Genetic differentiation between populations was not detected for species with samples collected from both the Mekong and Chao Phraya River systems, although several private haplotypes were observed in low frequencies in Chao Phraya and Mekong Rivers for *Pangasianodon hypophthalmus* and *P. larnaudii*.

Inference of population history

As there was no evidence of genetic differentiation, all natural samples within each species were pooled as a single group to conduct tests of selective neutrality and demographic history as for intraspecific diversity. Results of pairwise mismatch analysis and Tajima's D test performed on each species are given in Fig. 2. D values obtained from Tajima's D tests were negative and ranged from −2.245 for *P. larnaudii* to −1.103 for *Pteropangasius pleurotaenia*. These negative values (indicating more rare nucleotide site variants than would be expected under a neutral model of evolution) can result from selection and/or population expansion. Except for three species, that is *P. conchophilus*, *P. macronema* and *Pteropangasius pleurotaenia*, the hypothesis of neutral evolution was rejected with Tajima's D test (Fig. 3).

Table 3 Number of mtDNA 16S rRNA region haplotypes, number of haplotypes (*H*), number of polymorphic sites (PS), haplotype diversity (*h*), nucleotide diversity (π) and parameters estimated under the sudden expansion model

Species	*n*	*H*	PS	$h \pm$ SD	$\pi \pm$ SD	τ	*t*	*T*
Pangasianodon gigas	16	4	4	0.350 ± 0.148	0.0009 ± 0.0008	2.065	90095.99	0.90–1.44
Pangasianodon hypophthalmus	95	8	7	0.322 ± 0.061	0.0006 ± 0.0006	0.927	40445.03	0.08–0.20
Pangasius bocourti	42	10	8	0.576 ± 0.086	0.0013 ± 0.0011	0.908	39616.06	0.16–0.20
Pangasius conchophilus	26	3	2	0.145 ± 0.089	0.0003 ± 0.0004	3.000	130890.05	0.52–0.65
Pangasius larnaudii	217	11	13	0.133 ± 0.031	0.0003 ± 0.0004	3.032	132286.21	0.53–0.66
Pangasius macronema	17	2	1	0.118 ± 0.101	0.0002 ± 0.0003	3.000	130890.05	0.52–0.65
Pangasius sanitwongsei	65	6	8	0.345 ± 0.069	0.0008 ± 0.0007	3.000	130890.05	0.79–0.92
Helicophagus waandersii	55	7	6	0.324 ± 0.090	0.0007 ± 0.0007	1.013	44197.21	0.18–0.22
Pteropangasius pleurotaenia	12	5	4	0.667 ± 0.141	0.0016 ± 0.0013	1.345	58682.37	0.23–0.29

τ, time since the population expansion measured in units of $1/2u$ generations, where u is the per-nucleotide rate of mutation (1% per Myr is applied in the present study) multiplied by the number of nucleotides in the sequence; *t*, time since expansion in number of generations; *T*, time since population expansion in Myr]; SD, standard deviation

Distributions of pairwise differences between alleles of each species were compared with the pairwise mismatch distribution (Fig. 2) obtained under the sudden population expansion model (Rogers, 1995). Pairwise mismatch distributions for almost all species in this study conformed to Rogers' (1995) model of sudden expansion ($P =$ 0.052–0.542), except for that of *Pangasianodon hypophthalmus* ($P = 0.045$). A unimodal mismatch distribution was observed in all species, and all species showed a high proportion of paired comparisons between identical haplotypes (zero sites difference). Estimated possible population expansion times of the nine catfish species are shown in Table 3.

Interspecific relationships

K80 + *G* (equal base frequencies, transition/transversion ratio = 3.1268, γ distribution shape parameter $G = 0.1293$) was selected as the most suited model for the 16S rRNA sequences of pangasiid catfish. MP recovered a single most parsimonious tree ($L = 130$), which is identical to the tree recovered from ML analysis in terms of topology, with minor differences in bootstrap support at some nodes (Fig. 4). The tree recovered from NJ has a different topology with regard to the position of *P. bocourti* and *P. conchophilus* (Fig. 4). Overall, haplotypes within each species are clustered together with high bootstrap support (81–100%), whereas the confidence limits of interspecific relationships are rather poor at some nodes (Table 5).

Table 4 Pairwise F_{ST} between samples (MK, Mekong River basin; CP, Chao Phraya River basin; CS, captive stocks) of three pangasiid species examined based on 1000 permutations of the 16S rRNA sequences

Species	Origin	MK
Pangasianodon gigas	CS	(0.376)*
Pangasianodon hypophthalmus	CP	0.027*
Pangasius larnaudii	CP	0.000

Parentheses indicate significant F_{ST} values, while asterisks indicate that the exact test of allele frequency homogeneity is rejected.

Discussion

Genetic variation and historical demography

The present study reveals several significant findings in relation to the genetic diversity of the nine pangasiid catfish species investigated, including the critically endangered Mekong giant catfish. In general, low levels of intraspecific variation were observed not only in the critically endangered Mekong giant catfish but also in other closely related species that are presently common and abundant.

In general, it is predicted that genetic variation within species should positively correlate with population size, and as a consequence genetic variation in endangered species is expected to be lower than in non-endangered species (Frankham, 1996). In addition, genetic variation in body size relationships is often negatively correlated, and proven to be significantly so in mammals (Wooten & Smith, 1985; Frankham, 1996). The results from the present study, however, did not conform to the above predictions. *Pangasianodon gigas* is the largest freshwater fish in the Mekong; however, the observed haplotype diversity and nucleotide diversity of this relatively small natural population sample appear to be commensurate with that observed in other related species. Other studies on a range of endangered species have also shown similar results (e.g. Lewis & Crawford, 1995; Ge *et al.*, 1999; Gitzendanner & Soltis, 2000; Madsen *et al.*, 2000). This lack of correlation may be a result of the complicated processes involved in determining genetic variation at specific loci.

The unexpected, relatively high number of haplotypes observed in the present population of Mekong giant catfish could be a reflection of large historical population size. This genetic signature of large historical population size is likely reflected in current individuals for a long time due to the long generation time of this species (10–16 years in captivity; Meng-Umphan, 2000), as in the case of an endangered population of rhinoceros *Rhinoceros unicornis* in Chitwan Valley (Nepal) (Dinerstein & McCracken, 1990).

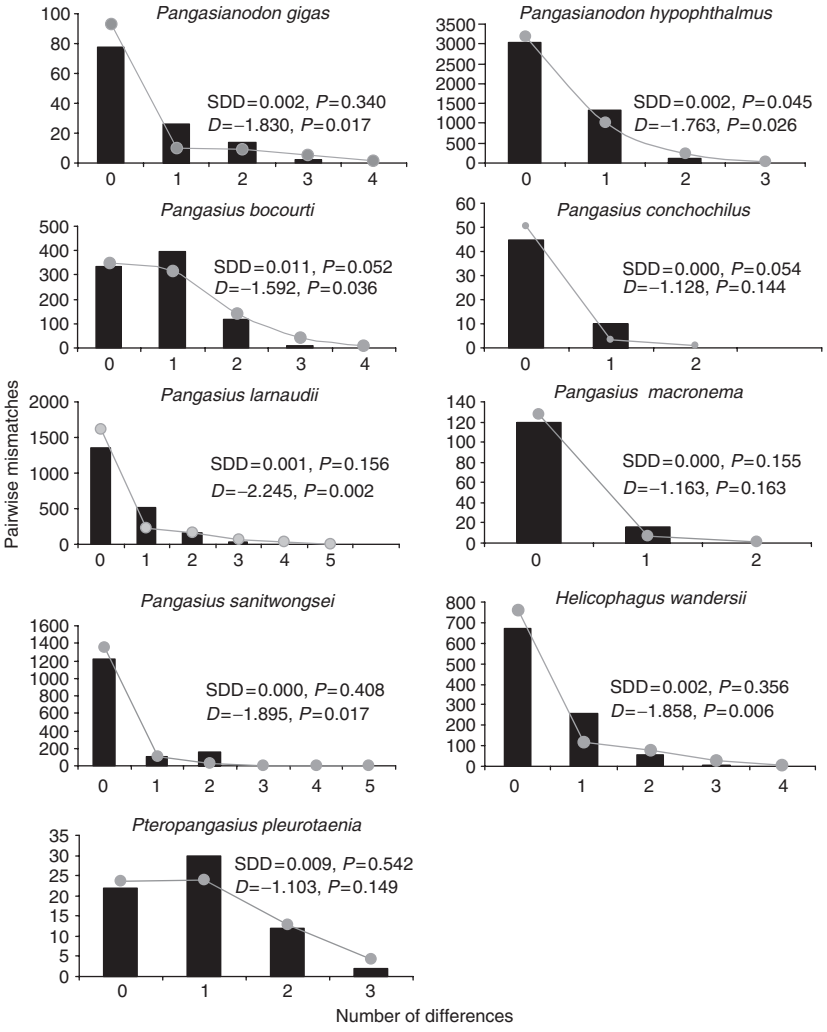

Figure 3 Results of mismatch distribution analysis using 16S rRNA sequences obtained from wild populations of the nine Pangasiid catfish species. Grey lines correspond to expected mismatch distributions. SDD, sum of squared deviation of mismatch distribution; *D*, Tajima's *D* value; *P*, probability.

The results of mismatch distribution and the neutrality test suggest that many species have undergone recent demographic expansion. Nearly all species appear to be in a mutation-drift disequilibrium. It is estimated that the possible times of expansion (Rogers & Harpending, 1992) for the Pangasiid catfishes range from 0.08 to 1.44 Myr ago. This implies that the expansion of these species occurred in the early to mid-Pleistocene. In the last 0.25 Myr, it is estimated that in this geographical region the sea level has been 75 m below the present level for up to 37% of the time (Voris, 2000). These geological events could have shaped genetic variation of the aquatic fauna in the region, and the catfish species studied may not be an exception in this regard.

It is apparent that the indication from genetic information in the present study is not in accordance with the available fisheries statistics. Although genetic data indicate an expansion of the populations of all species, fishery data report a significant decline in catches (Sverdrup-Jensen, 2002). A possible explanation for this conflicting observation is that the genetic data information presented here may reflect the genetic signature of past population(s) but not its present status.

Admittedly, in the case of the Mekong giant catfish, which was never caught in large numbers since the time records became available, the current catches are very few in number. For example, in the Cambodian sector of the Mekong River only 46 fish have been caught between 1999 and 2005 (Hortle *et al.*, 2005). In the Thailand sector there has been a significant decline in the number of giant catfish caught per year, from a high of about 40–50 fish in 1930 to an average of three fish in the period 2000–2005 (N. Sukumasavin, pers. comm.). This decline in catches may not only necessarily reflect a decline in population size but may also be due to behavioural changes, including the migratory pattern of the species, among other factors, which still remains largely unknown.

Implications for conservation

The level of mtDNA 16S rRNA sequence variation of the wild population of the critically endangered Mekong giant catfish is similar to most of its closely related species, except for *P. bocourti* and *Pteropangasius pleurotaenia*, which showed a greater level of diversity. It is important that conservation efforts should develop a strategy so that the current level of genetic diversity of the Mekong giant catfish is maintained over time.

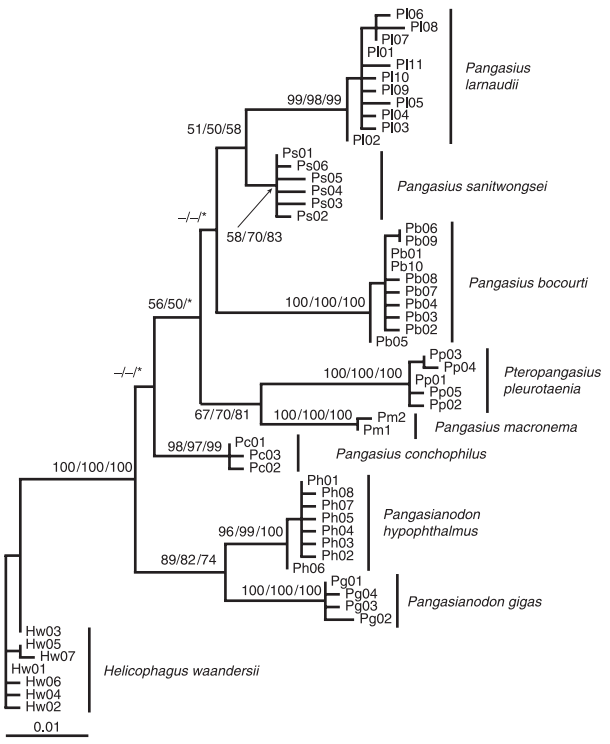

Figure 4 Maximum likelihood (ML) tree showing the relationships among 56 mtDNA 16S rRNA haplotypes from nine pangasiid catfish species. The numbers at each node represent bootstrap proportion based on 500 pseudoreplicates for ML and 1000 for maximum parsimony and neighbor-joining (NJ) analyses, respectively. – indicates that bootstrap values are lower than 50. *indicates that topology was different in the NJ tree.

Almost all fish samples of *Pangasianodon gigas* used in the present analysis were derived from wild parents captured in the commercial fishery, from which eggs and sperm were stripped for artificial fertilization. In almost all instances, stripping and the stress of capture of these large fish lead to mortality. It is believed that these fish contributed their genetic material to the present captive stock, which is currently held in a number of hatcheries in Thailand. However, the present analysis of 127 hatchery-bred individuals did not correspond to the level of genetic variation observed in the wild counterparts. The captive broodstock population is thought to be a critical resource for future efforts to rebuild the wild population(s). Thus for conservation purposes, it is important that a broodstock management plan that takes into account the process of founding broodstock be developed, and should include all available haplotypes to maximize the effective population size in order to maintain the genetic integrity of *Pangasianodon gigas* in captivity.

To date, most conservation efforts have concentrated on the Mekong giant catfish alone and little attention has been paid to its relatives. Species such as *P. sanitwongsei*, which is considered to be relatively rare in the Mekong and thought to be extinct in the Chao Phraya River, also deserves attention. In fact, it has been included in the IUCN Red List but as 'data deficient' (Poulsen *et al.*, 2004), and currently a strategy does not exist to preserve this species.

Captive breeding programmes for *Pangasianodon gigas* were initiated in 1984 with the aim to replenish depleted wild stocks. Currently, there are over 20 000 individuals of the first generation of *Pangasianodon gigas* in captivity. In general, most management strategies focus on the maintenance of a maximum level of genetic diversity of broodstock to ensure minimal adverse genetic impacts on wild counterparts after restocking or incidental escapements, as revealed in many other studies (Waples, 1991; Hughes *et al.*, 2003). With respect to *Pangasianodon gigas*, although the samples analysed may not represent the entire captive population, the common haplotypes seem to dominate the stock and therefore care must be taken in selecting broodstock for restocking purposes. For species such as *Pangasianodon hypophthalmus* and *P. bocourti*, which are not widely cultured in Thailand but mass produced elsewhere in the

Table 5 Summary of percentage sequence divergence within (diagonal) and between (below diagonal) the nine pangasiid species and calculated based on the K80 + *G* model

Species	PG	PH	PB	PC	PL	PM	PS	HW	PP
Pangasianodon gigas (PG)	0.004								
Pangasianodon hypophthalmus (PH)	0.024	0.004							
Pangasius bocourti (PB)	0.041	0.041	0.003						
Pangasius conchophilus (PC)	0.027	0.030	0.034	0.003					
Pangasius larnaudii (PL)	0.038	0.035	0.037	0.033	0.005				
Pangasius macronema (PM)	0.039	0.038	0.035	0.034	0.039	0.002			
Pangasius sanitwongsei (PS)	0.036	0.033	0.030	0.026	0.022	0.028	0.005		
Helicophagus waandersii (HW)	0.038	0.037	0.040	0.030	0.037	0.040	0.034	0.004	
Pteropangasius pleurotaenia (PP)	0.046	0.043	0.042	0.039	0.041	0.030	0.031	0.043	0.003

lower Mekong, particularly in Vietnam (Trong *et al*., 2002), special attention is needed in designing breeding programmes so that genetic diversity is maintained and the risks associated with inbreeding are minimized. It is also acknowledged that maintaining genetic diversity alone does not ensure survival in the wild due to possible behavioural and genetic adaptations in captivity.

Further studies

The ultimate goal of conservation programmes is to identify and preserve the historical population structure and/or patterns of diversity within and between populations of species under consideration (Vrijenhoek, 1994). With respect to the nine pangasiid catfish species, information concerning the population structure of each species is currently lacking, especially on a finer scale, for example upstream and downstream and between tributaries in each river system. Although no genetic differentiation was detected between the Chao Phraya and Mekong samples of *Pangasianodon hypophthalmus* and *P. larnaudii*, it is not certain at this stage whether to consider fish from the two river systems to be of one single stock or not. This needs further clarification using extensive sampling and more variable genetic markers such as microsatellites, which are readily available for pangasiid catfishes (Hogan & May, 2002).

In the present study, levels of genetic variation were estimated based on only a single non-coding locus. However, recent studies have criticized the use of non-coding genetic markers in that these may not reflect the variation that is important to the fitness of the species in question (Reed & Frankham, 2001; van Tienderen *et al*., 2002; Bekessy *et al*., 2003). On the other hand, using markers that only target a small number of genes is risky when assessing the biodiversity of endangered species, especially if there is a threat to the species from genome-wide inbreeding depression (van Tienderen *et al*., 2002). As such, further assessment of levels of genetic variation of pangasiid catfish species, including the critically endangered Mekong giant catfish, using a combination of both coding and non-coding loci, may be warranted (Hasson & Richardson, 2005).

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