CHAPTER V

DISCUSSION

5.1.1 Conservation of Microsatellites

Microsatellite loci generally have ancient origins and are highly conserved. Mostly, primers disigned for one species can be used with closely related species. For example, conservation of microsatellite loci was observed among three genera from family *Ictaluridae* (Liu *et al.*, 1999). Microsatellite homologies between freshwater and marine species of turtle showed that microsatellites were conserved for at least 300 million years (FitzSimmons *et al.*, 1995). In fish, microsatellite homologies between agnathans and gnathostomes showed 470 million years of conservation (Rico *et al.*, 1996) while a microsatellite locus in lamniform sharks has been conserved for a billion years (Martin *et al.*, 2002). Another study showed that microsatellite were detected in Centrachidae, Peridae and Esocidae which diverged 65 to 150 million years ago (Neff *et al.*, 1999). In the present study, all of the 21 microsatellite loci isolated from green arowana were detected in the gold and red strains, indicating that these loci were highly conserved among the arowana. However, loci *D01*, *D11* and *D15* were undetectable in some of the red tail Malaysian gold arowana. This suggests that these loci have partially diverged even within the same strain. Hence, not all microsatellite loci in arowana showed sufficient conservation of priming sites. Null alleles have been frequently reported within populations. Null alleles can be found in up to 25% of loci and their frequencies can be as high as 15% (Callen *et al.*, 1993; Paetkau & Strobeck, 1995). Point mutations (Lehmann *et al*, 1996; Paetkau & Strobeck, 1995) and short deletions (Callen *et al.*, 1993; Ede & Crawford, 1995; Jones et al, 1998) at priming sites may cause null alleles.

5.1.2 Variability of Microsatellite Loci

5.1.2.1 Mutation Rate in Different Types of Microsatellite

It is documented that there are several forms of mutation bias. Weber and Wong (1993) found that tetranucleotide repeats in human are more mutable than di- or trinucleotide repeats and the mutation rate for tetranucleotides is nearly four times higher than for dinucleotides. However, other studies showed that dinucleotide repeats have the highest mutation rate, followed by tri- and tetranucleotice (Valdes *et al.*, 1993; Chakraborty *et al.*, 1997; Edwards *et al.*, 1992). In arowana, the number of alleles was higher at most of the dinucleotide loci than at the tetranucleotide locus, *D31*.

Locus *D33* which is an interrupted microsatellite, was one of the least polymorphic loci among the 21 loci screened. Apparently, interrupted microsatellite repeats have lower mutation rates than perfect repeats (Weber & Wong, 1993). Stabilization of microsatellites by imperfections might be due to a greater difficulty for the formation of mutational intermediates (e.g. hairpin structure) or alter intermediates so that they are more easily recognized and repaired (Bichara *et al.*, 1995).

5.1.2.2 Correlation Between The Mutation Rate of A Microsatellite and Its Length

In most cases, the average repeat number at a locus is directly proportional to the degree of length polymorphism, which means that long loci mutate more frequent than short loci (Weber, 1990). Some further investigations implied that the

mutation rate within loci is positively correlated with allele size (Premmer *et al.*, 1996; Schlötterer *et al.*, 1998; Crozier *et al.*, 1999). Most likely, replication slippage can occur at more locations in repetitive sequences with many repeat units (Ellegren, 2000). However, results of the present study showed that there was no great difference in length polymorphism between locus *D37* which has the longest repeat number with short loci, such as *D27* and *D32*. The study of Yue *et al.* (1999) also indicated that short loci were more variable than locus *D37*.

5.1.2.3 Effects of Different Procedures

A direct comparison of the alleles found in the present study with those of Yue *et al.* (1999) showed that some correction of allele size was needed. In the present study, allele size was estimated using size fractionation during electrophoresis with Metaphor agarose gels whereas those of Yue *et al.* (1999) were estimated using denaturing polyacrylamide gels. This study showed that different laboratory procedures resulted in slight different estimates of size for the same allele.

5.1.3 Loss of Genetic Variability

The overall microsatellite variability in this study was substantially less compared with those estimated by Yue *et al.* (1999). Every strain showed a marked reduction of number of alleles, H_o and H_e values compared to the previous study. The number of alleles at each locus estimated by Yue *et al.* (1999) ranged from 5 to 22 whereas only 2 to 8 alleles were observed at each locus in the present study. The H_o and H_e values ranged from 0.28 to 0.84 and 0.32 to 0.95 respectively for the previous study. In this study, H_o and H_e values ranged from 0 to 1 and 0.17 to 0.80 respectively. The reason of low microsatellite variability in this study was most of the sample strains

were collected from a single population. On the other hand samples of Yue *et al.* (1999) were obtained from a few unknown sources (Orban, personal communication) and this contributed to a wider genetic pool.

The average number of alleles per locus for freshwater fish was 9.1 using polyacrylamide gels (DeWoody & Avise, 2000) while the estimates in the present study only ranged from 1.7 to 3.7 only. Low average number of alleles per locus was found in arowana, by other researchers. The ranges of 2.54 to 2.73 and 2.38 to 2.75 alleles per locus were detected although samples were run on polyacrylamide gels (Rahman, unpublished data, Sivananthan, 2003).

Large-scale sequencing of electromorphs will uncover size homoplasy. Large electromorphs uncover more hidden alleles than short ones (Viard *et al.*, 1998) since larger electromorphs are less stable to mutation than shorter ones (Primmer *et al.*, 1996). Size homoplasy within populations will slightly increase the average number of alleles (Viard *et al.*, 1998). More alleles can be present than alleles detected using DNA length variation alone. Therefore, individual heterozygosities may be underestimated with conventional microsatellite assays (Ortí *et al.*, 1997).

5.1.4 HardyWeinberg Equilibrium

A significant reduction of H_o was observed in the hatchery strains including the red tail Malaysian gold, red, yellow tail Malaysian gold and Indonesian gold arowana. Among the 21 microsatellite loci surveyed, there were large discrepancies between the H_o and H_e values for nine loci. These loci showed significant departures from Hardy Weinberg equilibria. Null alleles or inbreeding could be the reasons for

heterozygote deficiencies. Point mutations within the priminig sites, large insertion/ deletion between the repeat and the priming site of mutation within the repeat leading to large changes in product size may be responsible for the null allele (O'Connell & Wright, 1997). If an individual is homozygous for the null allele, no PCR product is observed. However, a null allele in a heterozygote may not be detected. This may be responsible for the deficit of heterozygotes. This type of 'partial null allele' phenomenon is common in cold-water fish microsatellites (O'Connell & Wright, 1997). High null alleles frequencies of 0.385 to 0.678 were reported in populations of Chinook salmon (Scribner *et al.*, 1996). The presence of a null allele at a frequency of about 0.100 would account for the heterozygote deficiency (Reily *et al.*, 1999).

The wild strains showed substantial reductions of allelic diversity in terms of the number of alleles per locus. The classical explanations for a reduced variability in a population include purifying selection, sampling error or a recent population bottleneck (Kirchman *et al.*, 2000). However, McDonald and Potts (1997) suggested that microsatellites are selectively neutral. Hence, it is unlikely that selection caused decreased variation in arowana. Sampling error seems to be possible. Only six wild green arowana and seven wild yellow tail Malaysian gold arowana were collected. Most likely the alleleic diversity depends on the sample size since the sample size of the wild strains was less than for the hatchery strains. Another possible explanation is that the samples selected may not be a true representation of the strain. Firstly, half of the total scale samples were collected from dead fish. Secondly, juvenile fish were not included as the scale-removing method might be harmful to them.

The sample size of the farmed green arowana was higher than those of the red and Indonesian gold arowana but the allele numbers detected were lower. This was caused by the loss of many rare alleles. Amos *et al.* (1996) suggested that if a population is large and expanding, the proportion of heterozygous individuals and the probability of a mutation occurring and being maintained in the population is increased. Changes in allele numbers are influenced by sample size whereas observed changes in heterozygosity are less influenced by sample size (Elliot & Reily, 2002). The variability at the microsatellite loci of the wild strains must be considered cautiously because of the limited sample size. This must be confirmed in the future by studies using larger sample size.

5.1.5.1 Population Bottleneck

Although the allelic diversities of the wild and reared green arowana and wild yellow tail Malaysian gold arowana were less than those for other strains, there were no significant reductions of H_e values. The F_{IS} values showed that these strains were in Hardy-Weinberg equlibria. Usually, loss of allelic variation without reduction in heterozygosity had been reported for hatchery strains. This phenomenon was observed in previous genetic assessments of farmed strains of turbot (Coughlan *et al.*, 1998), Atlantic salmon (Norris *et al.*, 1999), common carp (Devignes *et al.*, 2001) and sea trout (Was & Wenne, 2002). Most probably these strains were founded by heterozygous parents and the loss of low-frequency alleles might have little effects on heterozygosities (Allendorf, 1986). Changes in allele numbers are more sensitive for measuring changes in genetic variation. Populations that have recently undergone bottlenecks are likely to lose rare alleles but may still contain substantial amounts of heterozygosity. This is because rare alleles contribute less in

the estimation of heterozygosity (Nei & Roychoudhury, 1978). The observed number of alleles is less than the number predicted from the Hardy-Weinberg heterozygosity expectation under the assumption that the population is at mutation-drift equilibrium. Populations lose genetic variability after a bottleneck but as soon as the population size becomes large, variability starts to increase due to new mutations. The alleles number is reduced first, and than it recovers slowly. However, the average number of alleles per locus increases faster than the average heterozygosity after a bottleneck (Nei *et al.*, 1975).

The significant reduction of microsatellite variabilities detected in the wild green arowana might be caused by a population bottleneck. According to Briscoe *et al.* (1992) and Frankham (1995), genetic bottlenecks of less than 20 individuals are acceptable for many wild and captive populations because the genetically effective size (N_e) of a population is often 10 to 20% or even smaller of a population's census size. Although the wild green arowana are more abundant than the gold arowana and their distribution is wider in Peninsular Malaysia, their genetic variability was significantly much lower than that of the gold arowana. The larger population of the wild green arowana seem to experience a genetic bottleneck in the absence of a demographic bottleneck. A low fecundity rate and over exploitation may be the main factors for the reduction of population size in the wild. Since the green arowana fetch the lowest price compare to the red and gold strains, it breeding programme is not extensive. The hatchery strain of green arowana might be founded using wild green growana and no immigrant was introduced to this strain. Inbreeding was not the main reason causing a population bottleneck because the level of inbreeding was low and the F_{IS} value estimated for this strain was not significant. Occasionally,

small sample sizes may miss alleles present at low frequencies (Sjogren & Wyoni, 1994) thus resulting in allele frequency distributions that may resemble those from bottlenecked population. Therefore, more samples may be needed to avoid mistakenly identifying a nonbottlenecked population as a recently bottlenecked one.

The farm populations of arowana had increased quickly because of the lack of predators. Besides, farm bred arowanas mature faster than their wild counterparts due to better diet. Records for the establishment of arowana hatcheries are poor. There is lack of information on the contributions of the initial broodstock to the population and detailed records on the population over time. My results suggested that inbreeding did occur since the F_{IS} values showed that the farm bred red and gold strains had high inbreeding levels. Generally, genetic variabilities in hatchery broodstocks are lower than those in wild populations. This was observed in brown trout and rainbow trout (Gyllensten & Wilson, 1987). It is believed that the arowana hatcheries started with small numbers of broodstocks due to the high price of the gold and red arowanas. Besides, wild individuals of red and Indonesian gold could not be imported after the implementaion of the CITES regulations. Moreover, the aquacultural practice of selecting commercially important traits may further reduce the genetic variation. However, low breeding numbers might not constitute serious bottlenecks in red, Malaysian red-tail gold, Malaysian yellow-tail gold and Indonesian gold arowanas since there was no statistical support for such events.

There are a few explanations for the non-significant results I obtained when testing for the occurrence of bottlenecks in the gold and red strains. Recent immigration might occur to the hatchery strains since the breeders might increase

their broodstock at different stages. This could be misleading especially if the immigrants came from a population that was genetically divergent and they could quickly increase the number of rare alleles in the population without affecting the heterozygosity. As a result, recent immigration could mimick an increase of population size (Cornuet & Luikark, 1996). Furthermore, the F_{IS} values of the farm bred gold and red strains indicated the presence of null alleles at some loci. This phenomenon was very obvious in the Malaysian red-tail gold arowana. The partial null alleles could hide an excess of heterozygosity. The sample size of the wild Malaysian yellow-tail gold arowana might be too small to enable the detection of the bottleneck. In this case, this sample size was the best that could be obtained. Sampling of gold arowana was difficult in the wild due to over exploitation.

5.1.5.2 Importance of Genetic Management in Arowana Conservation

Many wild populations around the world are suffering from demographic and genetic bottlenecks due to habitat fragmentation and insularization (Pimm *et al.*, 1989). Bottlenecked populations may suffer from inbreeding depression, loss of genetic variation, fixation of deleterious alleles and increased demographic stochasticity (Lande, 1994; Mills & Smouse, 1994; Frankham, 1995). Furthermore, reduction in genetic diversity had been linked to decreases in growth and fecundity, changes in sex ratio and the ability to adapt to environmental changes (Chapman *et al.*, 1999). It is important to identify populations endangered due to genetic and demographic factors.

Loss of genetic variability in hatchery strains is usually caused by various factors, such as small initial breeding number, inappropriate mating designs, genetic

drift, inbreeding and selection (Winkler *et al.*, 1999). Maintenance of genetic variation is essential for the long-term survival of populations. The extent of variation determines adaptation to environmental changes (Fisher, 1958). The solution to minimize the genetic impacts of stocking to hatchery strains is to improve the genetic management by means of monitoring the genetic variability and estimating precise effective population sizes. Cross *et al.* (1993) suggested that if inbreeding can be avoided in a breeding programme, genetic variation may not be reduced despite small effective population sizes. This can be achieved by using a breeding programme based on pedigree information. Parentage analysis using molecular markers should be an ideal approach for these purposes.

5.2 MtDNA Variation

The results showed that most of the substitutions were concentrated at certain sites. This indicated that different sites have different substitution rates and those most of the nucleotides are highly conserved among the different arowana strains.

5.2.1 Transitional and Transversional Bias

Usually, newly arising transitions (TS) will outnumber transversion (TV) (Avise, 1986). This is because nucleotide transitions occur more frequently and accumulate faster than transversions in nuclear (Li *et al.*, 1984) and mtDNA (Aquadro & Greenberg, 1983). Previous studies of mtDNA sequences from fish species showed bias toward TS. This bias appeared to be weak relative to that found in mammals (> 10:1) (Brown, *et al.*, 1982), but another report of bias in *Osmerus* was as high as 11:1 (Taylor & Dodson, 1994). The TS:TV ratio of the control region (CR) in guppy was 3:1 (Fajen & Breden, 1992) while the ratio of the *ATPase* subunit

was 7.5:1 (Hurwood & Hughes, 1998). The TS-TV bias of cytochrome b in *Salmo* was 7:1 (Patarnello *et al.*, 1994). In rainbow fish, the TS outnumbered TV at all levels of divergence for cytochrome b, with values ranging from 1.2:1 to 2.3:1. For the CR sequence, a bias toward TS (2:1) was observed in comparison to closely related mtDNAs, but between the more divergent sequences, a bias toward TV was observed (Zhu *et al.*,). In the present study, a slight bias toward TS was observed between different colour strains of arowana. The ratio of TS : TV was 2.18 : 1. However, TV outnumbered TS between two Malaysian red-tail gold haplotypes. At high levels of divergence, when the number of TS does not increase much beyond a certain level even with increasing overall divergence, a "plateau" effect occurs (Irwin *et al.*, 1991). TS can also be "saturated" when additional TS does not increase the degree of divergence even if TS continues to happen (Brown *et al.*, 1982). The high ratio of TS : TV is probably caused by a low "ceiling" of sequence divergence and saturation of TS (Zhu *et al.*, 1994). The saturation of TS will reduce the informativeness of this region for resolving deeper relationships because additional transitions do not contribute to increased divergence. Hence, an effective transition-transversion weighting scheme should be applied to obtain an accurate topology for the arowana.

5.2.2 Conservation of Amino Acid Sequence

According to the universal genetic code, only about 3% of transitions at the third codon positions cause amino acid replacements whereas 41% of transversions alter the amino acid sequences. Nearly all substitutions at the first and second positions, regardless of whether they are transitions or transversions cause amino acid replacements (Wakeley, 1996). The results revealed that nearly half of the

substitutions occurred at the first and second positions, indicating that the non-synonymous substitution rate is high among arowanas. Besides, the ratio of the rate for synonymous versus non-synonymous is higher in *ATPase8* than in *ATPase6*, suggesting that these two different genes have experienced different selective pressures.

5.3 Genetic Differentiation

5.3.1 Correlation between Molecular Markers and Geographic Regions

The dendrograms derived from microsatellite and mtDNA data showed that the correlations between molecular marker diversity and geographic regions were weak. Although the green and the two strains of Malaysian gold arowana were found in Peninsular Malaysia, the Malaysian red-tail and yellow-tail gold arowana were closer to the Indonesian gold arowana from Sumatra. In addition, microsatellite data showed that the red arowana from Kalimantan had the closest relationship with the Indonesian gold arowana although Kalimantan is further away from Sumatra than Peninsular Malaysia. Sun *et al.* (1999) suggested that geographically close habitats can be different and conversely, geographical distant habitats can be similar in their environmental conditions. Geological evidence shows that continental South East Asian terrains can be classified into two categories, based on their Late Palaezoic tectonic history. The Shan Thai block which includes western Peninsular Malaysia and eastern Sumatra is characterised by Gondwanan affinities. This terrain is dominated by terrigenous, tilloid-bearing sediments in the lower part and carbonate formation in the upper part. On the other hand, eastern Peninsular Malaysia shows Cathaysian affinities and is characterised by predominantly tuffaceous, sandstones and siltstones (Hutchison, 1993). Hence, microsatellite variation, if under natural

selection, can display low correlations between populations separated by geographical distances.

5.3.2 Correlation between Molecular Markers and Quantitative Trait Variation

There are some slight conflicts between the results based on microsatellite and mtDNA data. Microsatellite data indicated green arowana as being the outgroup among the arowana strains and the Malaysian red-tail gold was closely related to the green arowana than to the farmed Malaysian yellow-tail gold and Indonesian gold arowana. In the present study, the genetic differentiation between each colour strain was significant, but the correlation between the topology of the microsatellite gene tree and the colour varieties was weak. This meant that the statistical significance of microsatellite loci only partially reflected biological realities. On the other hand, the topology of the mtDNA gene tree matched the different colour types of arowana. The mtDNA data indicated that the red arowana was the outgroup and this strain was closely related to the Malaysian red-tail gold arowana. The relationship between the gold strains was not confidently resolved. MtDNA or nuclear DNA genealogy may not be discordant with biological species boundaries due to demographically influenced stochastic patterns of lineage survivorship (Avise, 1986). Reed and Frankham (2001) suggested that molecular measures can only explain 4% of the variation in quantitative traits. There is a problem when comparing genetic differentiation derived from molecular markers with morphological traits. The microsatellite F_{ST} in this study should be quite accurate because 21 loci were used. Unfortunately, the loci for colour had yet to be determined. Furthermore, neutral molecular markers are inefficient for predicting patterns of variations in quantitative traits when selection was the main force. In this case, important morphological traits

such as colour variations show high levels of differentiation between strains. This showed that there were strong selective forces between colours but selection in other instances might not be so strong. Since there is no reproductive isolation among the strains, it is difficult to determine meaningful biological differences.

5.3.3 Genetic Differentiation among Strains

Other genetic markers such as RAPD and RFLP had been used previously to differentiate the various strains of arowana but the results were not promising (Fernando *et al.*, 1997). By using microsatellites, values of genetic distances were obtained yielded clear divisions among the different colour strains of arowana. This means that microsatellites are more effective in studying intraspecific relationships.

Husband and Barrett (1991) suggested that the small number of founders and their high relatedness would lead to greater genetic differentiation among founing groups but immigration could reduce these initial differences. It could be safely assumed that there was no hybridization the between different colour strains as each strain was endemic to certain river systems. Besides, hatcheries only produced pure breeds. These should be the main reasons for the significance of the genetic differentiation.

The wild and reared Malaysian yellow-tail gold arowana exhibited non-significant differentiation between them as half of the farmed Malaysian yellow-tail gold strain was created from the wild strain only one generation ago. Besides, they are found in Bukit Merah Lake which is a fairly small drainage system relative to the Pahang, Terengganu or Johor Rivers. On the other hand the distribution of the green

arowana is wider than those of the gold arowana in Peninsular Malaysia. Green arowana are found in several river systems in Pahang, Johor and Terengganu. Thus, geographical separation of the ancestral strains could be a reason to explain the significant differentiation between the wild and farmed green arowana.

5.4 Comparison of Microsatellite and MtDNA

Sometimes nuclear DNA (such as microsatellite) based genealogy may not perfectly concordant with mtDNA genealogy because mtDNA data only reflect female ancestry since mtDNA is maternally inherited (Avise, 1986). The difference in the estimates of genetic differentiation between microsatellites and mtDNA data can be influenced by differences in effective population sizes since the effective population size for mtDNA is one quarter that of microsatellites.

Usually differences between the significance of nuclear and mtDNA data suggested male-biased gene flow (Johnson, 1989). In this case, all the arowana strains are highly endemic to certain river systems and no hybridization occurred in the hatcheries. Hence, male-bias is not proven and both male and female dispersal are limited.

Differences in mutation rates between microsatellites and mtDNA may also influence the results. In this study, microsatellite data yielded a better differentiation among the arowana strains. The results showed that microsatellite loci were highly differentiated among strains but the differentiation among the gold strains was not fully resolved when using mtDNA. Only limited variations in the sequences of the mtDNA haplotypes were observed among the five colour strains. The results of the present study showed that low variability in mtDNA need not necessarily mean low

levels of genetic variation in the nuclear genome. Most of the microsatellite loci were more variable than the mtDNA, indicating that microsatellites evolved faster than mtDNA.

Microsatellites have been widely used for population-level studies. However, their efficiency may be reduced for longer-term comparisons (Hedrick, 1999). This is because in some hypervariable loci, autopomorphy outweights simulates of synapomorphy (Kirchman *et al.*, 2000). This will lead to difficulties in discerning historical relationships among populations. Thus, a combination of microsatellite and mtDNA data may overcome this problem.

The main constrain of the mtDNA analysis in this study is that the results were based on an analysis of a single mitochondrial marker from a few individuals. The mtDNA haplotypes which appeared fixed in these small samples may be more diverse in a larger sample size.

5.5 Biogeography of Arowana

5.5.1 Origin of Asian Arowana

Although the Asian arowana are distributed in South East Asia, its ancestors might have probably originated from Gondwanaland. It is beleived that the ancesters of the Asian arowana diverged from the Australian arowana in the eastern margin of Gondwanaland during the Early Creatacous and the divergence time estimated by Kumazawa and Nishida (2000) supports this explanation. India separated from Gondwanaland during the Cretaceous era and drifted nothwards across the Tethys Sea and eventually collided with Tibet during the Eocene. After the collision, India

acted as a stepping-stone, anabling fauna and flora of Gondwanaland origins to reach Asia and disperse into South East Asia (Hutchison, 1989). Some biotic elements which may not currently be represented in India due to climatic changes are in abundance in South East Asia and Sundaland (Holloway, 1998).

Most of the South East Asia continental terrains had their origins on the margin of Gondwanaland. They derived from Gondwanaland during the Devonian to the late Early Permian ages (Metcalfe, 1990). Some smaller terrains such as Sikuleh, Natal and Bengkulu separated from Gondwanaland in the Late Jurassic and most of the South East Asian terrains had coalesed by the end of the Cretaceous era (Metcalfe, 1998). Another hypothesis was that the Asian arowana were transported on these small terrains (Kumazawa & Nishida, 2000). However, the history of the Gondwananian land masses in South East Asia is still unclear (Metcalfe, 1999). Since the present results can only explain the recent intraspecific divergence of arowana, the direction of dispersal of the arowana is still uncertain. Hence, both of the hypotheses sound reasonable.

5.5.2 Dispersal and Divergence of Arowanas in South East Asia

From 55 to 30 MYA (Paleocene through Late Oligocene), global sea levels remained higher than the present sea levels (Hutchison, 1989). It is unlikely that arowana could disperse in South East Asia during this period. From the Late Oligocene to the Pleistocene, there were a few times of low sea-levels and Sundaland attained its large land mass. In the Late Oligocene, the sea level was as low as 250m below the present level. From the Late Oligocene onwards, the sea level rose progressively to about 220m above the present day level in the Middle

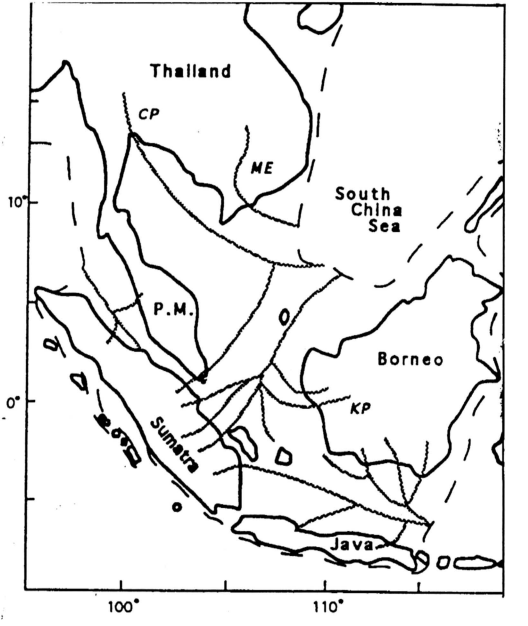
Miocene (13 MYA). Again in the Upper Miocene (10MYA) the sea level lowered to 220m below the present level. The estimated divergence time between the green, gold and red arowana (about 0.5 to 2.6 MYA) made it unlikely that these strains split during the Middle Miocene. At the Miocene-Pliocene boundary (5.2 MYA) there was a rise in sea level world-wide to about 140m above present sea level. This was followed by several cycles of rapidly fluctuating sea levels due to the many glacial and interglacials periods after the Pliocene (Hutchison, 1989). The estimated divergence time between the green and red arowana (1.5 to 2.6 MYA) is close to the probable time of the fluctuation of sea levels during the late Pliocene to the early Pleistocene.

It is believed that the different strains of arowana which now inhabit separate regions of South East Asia were connected through freshwater habitats during the Pleistocene (Goh & Chua, 1999). Assuming the sequence divergence rate for *ATPase* determined for fish of 1.3% per million years (Bermingham *et al.*, 1997) the divergence between the three strains of gold arowana occurred in the early part of the late Pleistocene. The haplotype sequences of these three strains only differed by less than 1%, indicating that dispersal events occured during the Pleistocene. Haplotypes within the red and green strains diverged in the middle of the Pleistocene while haplotypes within the Malaysian red-tail gold and the Indonesian gold arowana diverged during the late ice age. Divergence between haplotypes of the Malaysian yellow-tail gold arowana occured after the Pleistocene.

In South East Asia, geological evidences show that there were important sea level fluctuations, especially during the Middle and Late Pleistocene ages. Possibly

several times of Pleistocene period low sea levels of between 50 and 150 m occurred (Prentice & Denton, 1988). A huge contimental shelf called Sundaland was exposed. The Indonesian islands such as Sumatra, Java and Borneo were connected to mainland Asia and Peninsular Malaysia by land bridges (Heaney, 1985). On Sundaland there were several very large river systems. The Malacca Straits River system flowed between Peninsular Malaysia and Sumatra. The Siam River system joined Thailand's Chao Phraya River with the Endau, Pahang, Terengganu, and Kelantan Rivers of Peninsular Malaysia. The North Sunda River ran north from the north-east coast of Sumatra to join the Kapuas River of Borneo. The East Sunda River system included the rivers of the north coast of Java, the south coast of Borneo and the northern portion of the east coast of Sumatra (Voris, 2000). During the Pleistocene glacial maxima, the islands Sundaland and the mainland were connected by lowlands traversed by these rivers (Fig. 5.1). Each time this happened, there were fauna exchange among Peninsular Malaysia, Sumatra, Borneo and Java. During the glacial minima, these islands were separated due to the rising of the sea level. As a result, various of arowana strains diverged from the late Pliocene to the middle Pleistocene. A similar phenomenon happened to the river catfish (*Hemibagrus nemurus*) which is widespread in South-east Asia and evidence indicated that they colonized this region before or early during the Pleistocene rather than the results of dispersal during recent Pleistocene (Dodson *et al.*, 1995). Another widespread fish, the cyprinids are desendant of a single species that dispersed from Borneo to Midanao probably during the Pliocene (Kornfield & Carpenter, 1984).

Fig. 5.1. Location map of the contemporary river systems adapted from Verstappen (1975). Dashed lines indicate the edge of the Sunda shelf and the maximum extent of the Sunda lowlands during Pleistocene glacial maxima. Hatched lines indicate the major drainage systems proposed to have existed during the most glacial glaciation.



The migration route of arowana in Sundaland is unclear due to the lack of fossil evidence and records of the river system evolution. A hypothesis proposed that the Late Middle Pleistocene cooling drove the northern fauna to migrate southwards to the Indonesian islands. There was only an exceptional case of faunal exchange from Indonesia to the South East Asian mainland (Tougard, 2001). Further studies to detail the relationships among the populations are needed to understand the migration route of the arowana. Sampling should include populations from Kelantan, Terengganu and even Thailand.

5.5.3 Evolution of Arowana

The isolated populations evolved in allopatry at the intraspecific level. Dodson *et al.* (1995) reported that west Peninsular Malaysia has greater faunal affinities with Sumatra while east Peninsular Malaysia has greater affinities with the Indochinese fauna. This hypothesis is supported by the distribution of the Malaysian and Indonesian gold arowana in the west of Peninsular Malaysia and in eastern Sumatra respectively. Their close relationships revealed by mtDNA data also reflected the historical patterns of connections between the river systems. On the other hand, the green arowana are distributed in the east of Peninsular Malaysia, Thailand and Indochina. The Malaysian gold and green arowana are only found in west and east Peninsular Malaysia respectively. East and west Malaysia are separated by a central range of mountains but differences may only be manifested at the intraspecific level as was in the case of the river catfish, *Hemibagrus nemurus* (Dodson *et al.*, 1995).

Each colour strain exhibits high endemicsm to certain river systems. Even individuals within a strain but orginating from different rivers show some slight differences in colour shading. Some of the local bred red arowana exhibited a shade of orange after a few generations (Ng, K. H., personal communication). Since certain morphological traits may represent phenotypic adaptations of specific habitats (Lansman *et al.*, 1983), this leads to the question of what factors are responsible for the evolution of the various colours. However, there is lack of data to show that environmental factors, such as water quality and soil composition or different diet could influence the colour of arowana after many generations. Further studies are essential to clarify the evolution of the different colour strains.

5.6 Speciation of Arowana

The divergence between the two haplotypes of green arowana was 1.4% while the divergence between the two haplotypes of red arowana was 2.0%. This divergence was greater than the divergence among haplotypes of the three strains of gold arowana (0.2 to 0.5%). This showed that divergence within strains was greater than divergence between strains. The divergence between the green, gold and red arowanas ranged from 0.7% to 3.4% and they were diverged for 0.5 to 2.6 million years. However, they are considered as members of a single species. The *ATPase* divergence between *Prochilodus* species in South America ranged form 0 to 5% (Sivasundar *et al.*, 2001). These empirical data agreed with the hypothesis suggested by Klicka and Zink (1997) and Avise *et al.* (1998) that most of the speciation events might have occurred 4 MYA which was before the Pleistocene. On the other hand, genetic divergence of cytochrome b between species pairs of fish in the Africa lakes

ranged between 0 to 1%, indicating times of divergence of less than one million years (McCune, 1997; McCune & Lovejoy, 1998).

Engstrom *et al.* (2002) assumed that long-term reproductive isolation would be reflected in the divergence of genetic markers and morphologies within independent lineages. Although the various strains of arowana show genetic and morphology differentiation, they can interbreed. This means that reproductive isolation which leads to speciation according to the Biological Species Concept (Mayr, 1963) was not happened. Hence, although long-term genetic isolation may cause substantial genetic differentiation, it is not sufficient for any speciation events in arowana.