# THESIS

# DEVELOPMENT OF MOLECULAR MARKERS FOR IDENTIFICATION OF THAI CROCODILES

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GRADUATE SCHOOL, KASETSART UNIVERSITY

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# THESIS APPROVAL

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THESIS

# DEVELOPMENT OF MOLECULAR MARKERS FOR IDENTIFICATION OF THAI CROCODILES

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Agricultural Biotechnology)
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Win Chaeychomsri 2008: Development of Molecular Markers for Identification of Thai Crocodiles. Doctor of Philosophy (Agricultural Biotechnology), Major Field: Agricultural Biotechnology, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Voravit Siripholvat, Ph.D. 74 pages.

Microsatellite DNA marker was developed from a wild caught *Crocodylus siamensis.* Genomic DNA was extracted from whole blood cells and digested with *Taq* I, and DNA fragments in the range of 500 to 1000 bp were ligated into pBluescript II KS+ phagemids and transformed into fresh competent XL1-Blue *E. coli* host cells by heat shocking. Positive clones were transferred to Hybond N membranes and hybridized with synthetic oligonucleotide microsatellite probe d $(AC)_{15.}$ The ligated target-plasmid DNA was isolated and recombinants that containing microsatellite repeats were sequenced automatically. Primers were designed for microsatellite loci containing dinucleotide repeats. Sequences of 22 positive clones were submitted in GenBank (accession numbers EF413033-EF413054). Twenty microsatellite loci were evaluated in 5 captive breeding crocodile farm for species testing. Ten (50 % of total microsatellite primers) of the 20 microsatellite loci were polymorphic. These primers were successful across eighteen loci for the 40 crocodiles of 29 *C. siamensis*, 4 *C. porosus*, 5 *Caiman crocodilus crocodilus* and 2 hybrids. Fourteen microsatellite loci showed specific allele were considered as specific markers for identify species of crocodiles. These markers composed of CS-25 for identify *C. siamenis*, *CS*-4, *CS*-26 and *CS*-30 for identify *C. porosus*, *CS*-2, *CS*-7, *CS*-10, *CS*-12, *CS*-14, *CS*-17, *CS*-22, *CS*-24 and *CS*-35 for identify *C. c. crocodilus* and *CS*-20 that can identify all species. Twelve microsatellite loci showed polymorphic alleles in the same species that were considered as intra-specific markers. These markers composed of CS-4, *CS*-5, *CS*-10, *CS*-12, *CS*-17, *CS*-18, *CS*-21, *CS*-24, *CS*-26, *CS*-30, *CS*-32, and *CS*-35. Seven microsatellite loci showed absent of allele in *C. c. crocodilus* that were considered as genus-specific markers. These markers composed of CS-4, *CS*-5, *CS*-18, *CS*-21, *CS*-26, *CS*-30, and *CS*-32. These microsatellite loci can be used for species testing and also evaluated for assigning parentage in situations where maternity, paternity, or both may not be known. It is therefore recommended that more microsatellite markers be used in these situations. The use of these microsatellite markers will broaden the scope of a breeding program, allowing progeny to be tested from adults maintained in large breeding lagoons for selection as future breeding animals.

Win Chaeychomsri

Student's signature

Thesis Advisor's signature

1 / May / 2008

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May 2008

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# DEVELOPMENT OF MOLECULAR MARKERS FOR IDENTIFICATION OF THAI CROCODILES

## INTRODUCTION

The Siamese crocodile *(Crocodylus siamensis)* is a critically endangered species of freshwater crocodile that had a previous range throughout much of SE Asia. It was once widespread within the freshwater wetlands of Thailand (Ratanakorn *et al*., 1994). However, its is difficult to conserve because the people who have to live near them generally consider them to be dangerous and unattractive. Crocodile conservation is therefore focused upon very practical economic incentives. Successful management programs worldwide are based upon a sound understanding of crocodile biology, but many uncertainties remain that have proven intractable to field research techniques or direct observation.

Natural hybridization has been an important component in the evolution of a wide range of taxa (Arnold, 1997; Allendorf *et al.,* 2001; Barton, 2001) and in the interest of conserving biodiversity there is a recognized need to provide for the continuation of such evolutionary processes (Moritz, 1999). On the other hand, anthropogenic hybridization brings together species that would otherwise not bred due to lack of opportunity, when spatial or temporal isolation is eliminated or behavioral cues become disrupted by altered environments. Anthropogenic hybridization may be intentional, particularly for captive commercially used species, or it may result from the introduction of exotic species and habitat fragmentation or alteration. Unintentional hybridization is often a more serious conservation concern because it can occur undetected, particularly if hybrids do not have distinguishing morphological features. Intentional hybridization can become a concern however, if mixed species populations become the source for reintroduction efforts. What will be critical is the extent to which hybridization has occurred, whether the population consists mostly of purebred parents and F1 offspring, or whether there is complete admixture and a population consisting only of hybrids (Allendorf *et al.,* 2001).

Because mating occurs in the water and often involves groups of males and females which are difficult to differentiate, clear observation is rarely possible (Lang, 1989). Even if a female was mounted by more than one male, it is unclear whether multiple males successfully copulate and inseminate her, resulting in fertilized eggs. Such observations have led to the supposition that female crocodiles may produce clutches sired by multiple males. Multiple paternity the occurrence of offspring within a single clutch being fathered by more than one male, is a mating strategy known to be utilized by a variety of taxa, including horseshoe crabs, spiders, black bears and birds (Brockmann *et al.*, 1994). Among the reptilians, four orders have documented accounts of multiple paternity snakes (Zweifel and Dessauer, 1983), lizards (Abell, 1997), terrestrial and marine turtles (FitzSimmons, 1998) and crocodiles (Davis *et al.*, 2001).

Sustainable use is seen as an important aspect of conservation programs for many crocodilians (Ross, 1998) and the use of genetic markers to determine the extent of gene flow among populations may contribute to more effective programs. It is generally unknown what constitutes genetically discrete populations of a given species. Studies of genetic composition of individuals and of populations of crocodilians have the potential to answer many questions that will directly address management, regulation and conservation decisions. DNA analysis also has potential for application in captive breeding programs, evaluating hybridization, the effects of introductions of exotic species and to the identification of products in trade and reintroduction programs.

# OBJECTIVES

1. To design microsatellite primer from the construct libraries of wild freshwater crocodile.

2. To test which designed primers shown polymorphic DNA band in captive freshwater crocodiles.

3. To determine the DNA polymorphic from that can be used as specific markers to distinguish freshwater and saltwater crocodiles.

# LITERATURE REVIEW

## 1. Crocodilian biology

Crocodilians (crocodiles, alligators, caimans, and gharials), are prominent and widespread occupants of tropical and subtropical aquatic habitats. The group is of great antiquity with hundreds of fossil forms and three major Families. Table 1 shows the taxonomy of the extant 23 species. Crocodilians are implicated in positive effects in their environments as "keystone species" that maintain ecosystem structure and function by their activities (King, 1988; Craighead, 1968). These include selective predation on fish species, recycling nutrients, and maintenance of wet refugia in droughts.

Table 1 List of the species of crocodilians (King and Burke, 1989).

- Order Crocodylia
  - Family Alligatoridae
    - *Alligator mississippiensis* (American alligator)
    - *Alligator sinensis* (Chinese alligator)
    - *Caiman crocodilus* (caiman) includes *C. crocodilus crocodilus*, *C. c. fuscus*, *C. c. apaporiensis*, *C. c. chiapasius*
    - *Caiman latirostris* (broad-snouted caiman)
    - *Caiman yacare* (yacare)
    - *Melanosuchus niger* (black caiman)
    - *Paleosuchus palpebrosus* (dwarf caiman)
    - *Paleosuchus trigonatus* (smooth-fronted caiman)
  - Family Crocodylidae
    - Subfamily Crocodylinae
      - *Crocodylus acutus* (American crocodile)
      - *Crocodylus cataphractus* (slender-snouted crocodile)
      - *Crocodylus intermedius* (Orinoco crocodile)
      - *Crocodylus johnstoni* (Australian freshwater crocodile)

*Crocodylus mindorensis* (Philippine crocodile)
*Crocodylus moreletii* (Morelet's crocodile)
*Crocodylus niloticus* (Nile crocodile)
*Crocodylus novaeguineae* (New Guinea Crocodile)
*Crocodylus palustris* (mugger)
*Crocodylus porosus* (saltwater crocodile)
*Crocodylus rhombifer* (Cuban crocodile)
*Crocodylus siamensis* (Siamese crocodile)
*Osteolaemus tetraspis* (dwarf crocodile)
Subfamily Tomistominae
*Tomistoma schlegelii* (tomistoma)
Family Gavialidae
*Gavialis gangeticus* (gharial)

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Crocodilians have some unique aspects of natural history that create special challenges for their conservation. They are the largest predators in their habitats and can threaten humans and their livestock. Many species are exploited for their valuable skin, which supports an international trade worth over US$500 million annually. Habitat loss and the pollution of aquatic habitats also heavily affect them. Loss of any species of crocodilian would represent a significant loss of biodiversity, economic potential and ecosystem stability.

There is a wide diversity of size, habitat, food preference, reproductive behavior and many other aspects of biology among the 23 species of crocodilian. However, all species have the following basic similarities. All crocodilians are very effective aquatic predators. At smaller sizes they often eat aquatic insects, small fish and crustaceans and as they grow larger they tend eat more vertebrates, including fish, turtles, birds and mammals. Crocodiles attempt to maintain their body temperature within narrow limits by basking in the sun when cool and seeking shade when hot. They are metabolically efficient and have fast reflexes and effective locomotion ability on land, where they walk on erect legs, and in the water, where they swim rapidly driven by their powerful tails. Crocodilians have complex behaviors including social interactions, dominance hierarchies, vocalization, coordinated feeding, and well-developed maternal behavior. Females' deposit from 10 to over 60 hard-shelled eggs into a nest, which is either a hole, dug into the ground, or into a mound of vegetation formed by the female (Figure 1). Most females remain near their nest during incubation and may protect it from predators. Upon hatchling, vocalizations made by the hatchlings induce the female to assist the hatchlings to emerge, and in some cases to carry the tiny babies to the water in her mouth. Hatchlings remain together near the mother for several months, deriving protection from her. As they grow and become more widely dispersed and independent, a large number of the offspring perish some eaten by other crocodilians.

**Figure 1** Female deposit hard-shelled eggs into a mound of vegetation formed by the female and remain near their nest during incubation and may protect it from predators.

The survivors reach maturity after a period of 5-15 years depending on the species. Females grow more slowly and reach maturity at a smaller size than males, who continue growing and usually exceed females in maximum size. Crocodilians can be long lived in the wild and there are records of particular individuals residing for decades at some locations. Adults of several species emit loud vocalizations during the breeding season. These biological characteristics give the potential of great resiliency to some crocodilian populations, enabling them to recover from population depletion and sustain relatively high harvest rates. However, unregulated killing of adults can lead to rapid population depletion, particularly if combined with habitat loss.

## 2. Threats to crocodilians

Crocodilians are threatened by many human activities. Foremost and the most significant among these is the destruction or alteration of wild habitat. In the past, commercial overexploitation and indiscriminate killing have resulted in many species suffering drastic declines in numbers and reductions in distribution, but no species has become extinct because of direct human exploitation. However, over exploitation combined with severe habitat loss have brought several species to the brink of extinction.

Crocodilians of all species depend upon wetland habitats. Different species have varying preferences and requirements and crocodilians have adapted to most available tropical and subtropical wetland types (marshes, mangroves, rivers, lakes, lagoons etc.). Because they are quite large animals and because crocodiles increase through several orders of magnitude of size as they grow from hatchling to adult, they require areas of habitat that are both large and diverse. A few species are adaptable and are able to persist in small areas of disturbed habitat, for example the common caiman and Chinese alligator. However, most species require relatively large areas (hundreds of square kilometers) of undisturbed wetland to maintain large populations.

Habitat destruction has taken many forms. The most obvious destruction of wetlands is by drainage and infilling, deforestation, conversion to agricultural use and pollution. However, subtler habitat alteration may also be disastrous for crocodiles. In the Andaman Islands the saltwater crocodile is dependent upon restricted areas of fresh water marsh for successful nesting. Unfortunately these areas are increasingly used for human agriculture and crocodiles attempting to nest in them are killed. Therefore, while large areas of pristine riverine and mangrove habitat remains, the population is in decline. In the Philippines, people live along the rivers and use them extensively for fishing and transport. While the riverine habitat appears intact, and many areas have relatively low human densities, the constant attrition of crocodiles killed by people and caught in nets has caused the virtual disappearance of two species, the Philippine and saltwater crocodiles. Rural people are often intolerant of large and potentially dangerous crocodiles and the deliberate destruction of both nests and adults is widely reported (Madagascar, Andamans, China, Bangladesh). The creation of dams and impoundments has an ambiguous effect. Initially, the original complex, well vegetated marshy habitats may be replaced by simpler reservoir lakes with bare shores and crocodile populations may decline.

## 3. Crocodile in Thailand

There are three species of crocodiles in Thailand but only two species of crocodiles that usually culture in Thailand, Freshwater or Siamese crocodile (*C. siamensis*) and Saltwater or estuarine crocodile (*C. porosus*).

Freshwater or Siamese crocodile (Figure 2)

Siamese crocodile was once widespread within the freshwater wetlands of Thailand (Ratanakorn *et al.*, 1994). It can be seen in rivers, reservoirs and swamps especially at Bung Boraphet in Nakhornsawan Province and Bung Jorakae-Samphan in Uttaradit Province.

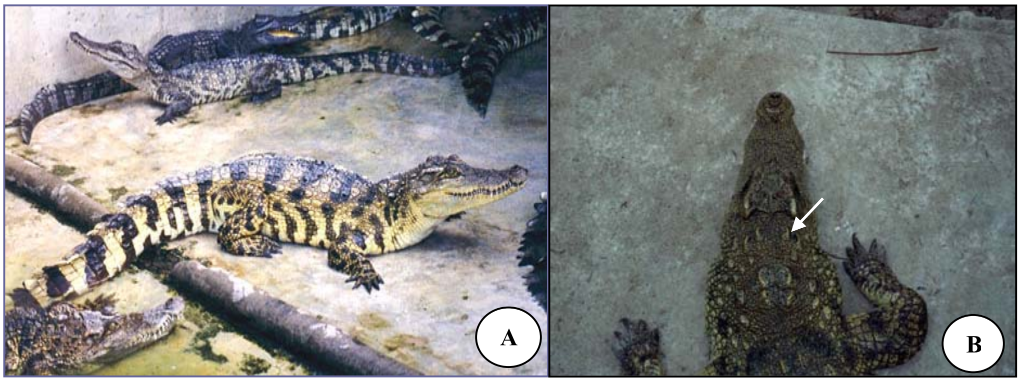

**Figure 2** Juvenile *C. siamensis* showed bright yellow color (A). Crocodile's head showed 4 posterior occipital knob (arrow) (B).

Food : Mainly fish, and sometimes other small crustacians.

Range: Thailand, Vietman, Cambodia, Lao PDR, Malaysia (Sabah, Sarawak) and Indonesia.

Characteristics: Males can grow up to 4 m, but most individuals do not exceed 3 m.

Biology: They reach maturity at 10-12 years of age. Females construct mound nests during the annual rainy season and lay 20-50 eggs. Incubation period is 68-85 days. This species is relatively unthreatening to people (compared to saltwater crocodile).

Saltwater or estuarine crocodile (Figure 3)

Saltwater crocodile can be seen in southern Thailand in brackish water habitat and the tidal sections of rivers. A great deal of ecological work has been done on this crocodile in Australia and New Guinea.

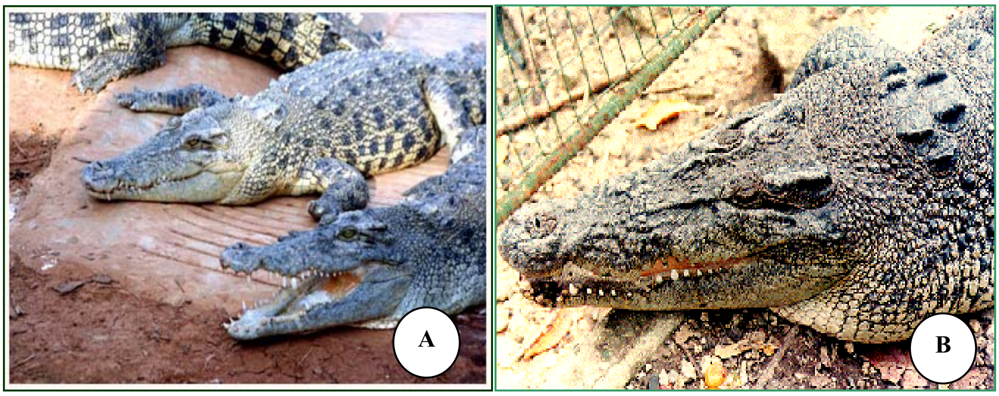

**Figure 3** *C. porosus* showed grey color (A). Crocodile's head lacked posterior occipital knob (B).

Food: Mainly fish

Range: Australia, Bangladesh, Brunei, Cambodia, China, India, Indonesia, Malaysia, Mynmar, Papua New Guinea, Philippines, Singapore, Sri Lanka, Solomon Islands, Thailand, and Vietnam.

Characteristics: This species is the largest of the living crocodile in the world. Their lengths were reported to reach 6-7 m. Noted for its large size and fierce disposition.

Biology: Males reach adulthood at 16 years of age while females become mature at 12 years of age (lengths of approximately 2.2-2.5 m) and make mound nests during the annual rainy period. Clutch size is typically 40-60 eggs and incubation period lasts some 90 days.

Thailand conducted a whole crocodile business from producing to marketing, including feeding, slaughtering, tanning, and making genuine crocodile leather products. We have 20 farms which have received permission from Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to sell crocodile products to both domestic and international customers. For this industry to meet the demand for a high quality product, the adoption of a genetic improvement program is essential. The program will be based on the selection of candidates based on their own and their relatives' phenotypic performances for selection criterion related to defined selection objectives. Self evidently, the implementation of a successful breeding program will require correct pedigrees. Errors in assigned parentage decrease the accuracy of genetic evaluation of candidates, resulting in realized genetic improvement being less than expected.

Only a small number of adult crocodiles on Thailand crocodile farms are kept in unitised breeding pens (1 male: 1 or 2 females). The majority of adults are maintained in breeding lagoons containing many males and females. In these situations, mating are neither observed nor recorded. It would be advantageous for industry, and for the success of a genetic improvement program, to include these animals and their offspring in a multi-trait selection program.

A common method of marking a crocodile for identification is to cut scutes in a unique sequence. Scutes are vertical triangular osteoderms on the dorsal midline of the posterior tail that bifurcate into two rows of more laterally flattened scales in about the middle third of the tail and continue cranially (Richardson *et al.*, 2002). In captive breeding, this method of marking is usually done on the day of hatch. This method, as described by Richardson *et al.* (2002), is one of the few permanent and practical identification methods available, although it is without problems. Errors can result from incorrect cutting of the scute sequence at hatch, incorrect reading of the scute numbers later, or numbers changing over time due to the regrowth of incorrectly cut scutes. Other methods include toe tagging and microchips, which have their own disadvantages.

## 4. Type of genetic markers

The use of genetic markers in aquaculture has increased over the last 20 years as they have been applied to many aspects of production and management. Several types of marker exist, the most commonly used include protein products (allozymes), mitochondrial DNA (mtDNA), variable number tandem repeats (VNTR) and randomly amplified polymorphic DNA (RAPD). Each method has distinct properties and generates different types of data for a variety of applications. The discovery of new markers has not replaced previous techniques and by using a combination of markers many of the problems in aquaculture can be resolved.

Protein methods can be summarized as the analysis of electrophoretically distinguishable protein variants. Allozymes (product of different alleles at the same locus) are one of the earliest and therefore most widely used type of markers in population-level fishery researches. The techniques of electrophoretic separation and staining are easily adjustable to different species and any source of soluble protein can be used (Magoulas *et al.*, 1998). Therefore, the use of allozymes has been widespread for population studies needing inexpensive, rapid stock identification on a large scale (for example, Ross, 1998). Allozymes can be particularly useful for analysing differences between species (Guiffra *et al.*, 1996). The method is, however, limited due to the sole focus on genes expressing proteins detectable with a stain, the restricted amount of polymorphism found in some species and the need for large tissue samples for analysis (Park and Moran, 1995).

Mitochondrial DNA (mtDNA) is characterized by high levels of sequence diversity at the species or infra-specific level. It is transmitted only through the mother, does not recombine and is present as multiple (and usually identical) copies within a cell. As a result of transmission through the maternal line, the effective population size of mtDNA is one-fourth that of nuclear DNA (Nei and Tajima, 1981). This allows population events such as bottlenecks and hybridizations to be detected more easily, but it cannot be used to detect male mediated genetic mixing of stocks (Magoulas *et al*., 1998). Different parts of the mitochondrial genome have been looked at for particular applications: the cytochrome b, D-loop and ND genes in population studies (Carr and Marshall, 1991; Park *et al.*, 1993; Nesboe *et al.*, 1999) and the ribosomal genes in species or even family-level studies (Geller *et al.*, 1993).

The main advantage in using nuclear DNA (nDNA) markers is that they are widely dispersed throughout the genome in non-coding areas that are not normally under as great a selective pressure as that found in coding sequences. This allows a rapid evolution of markers that can be used for identification to the species and individual level. The first nDNA markers developed were randomly amplified polymorphic DNA (RAPD) in which short primers of arbitrary sequence were used to isolate anonymous regions of DNA (Williams *et al.*, 1991). The technique is quick and also cost effective, as there is no need to look for target sequences or to design specific primers. The interpretation of the markers, however, is often difficult and unreliable. The main application for RAPD’s has been the comparisons between species and sub-species (Bardakci and Skibinski, 1994), for example stock identification (Gomes *et al.*, 1998; Elo *et al.*, 1997; Koh *et al.*, 1999) and biomarker experiments (Nadig *et al.*, 1998; Theodorakis *et al.*, 1998).

Repetitive DNA forms 10-30% of the genome and where this occurs as clusters, or blocks of tandemly repeated sequences, it is termed satellite DNA. The function of these blocks of repeat sequences is not fully understood, but the high frequency found in sub-telomeric areas and near centromeres suggests it may promote recombination or play a role in chromosome function (Twyman, 1998). Satellite DNA is highly polymorphic and markers can be divided into two types: i) minisatellite or variable-number tandem repeats (VNTR) - repeats of small length (10-64 base pairs); and ii) microsatellite - short tandem repeats (2-4 bp).

Minisatellites are the basis of ‘DNA fingerprinting’ and due to high degrees of mutation and heterozygosity in the sequences, they can be used to distinguish between individuals. This makes the markers ideal for pedigree analysis (Clifford *et al.*, 1998; Thomaz *et al.*, 1997) and stock identification (Beacham, 1996; Beacham *et al.*, 1996, Galvin *et al.*, 1996). However, minisatellites are not a good choice for population-level investigations as multiple loci are assayed simultaneously, allelic frequencies cannot be determined and the mutation rate is high (Magoulas *et al.*, 1998).

Microsatellite DNA markers are smaller and simpler than minisatellites, occurring as di-, tri- and tetra-nucleotide repeats. In addition, they are more numerous and widespread in the genome, with frequencies of $10^4$ to $10^5$ copies in fish (Park and Moran, 1995). Each microsatellite is usually flanked by unique sequences that can be used as primer binding sites for amplification of the microsatellite using the polymerase chain reaction (PCR) technique. An advantage of using PCR to assay the markers is that only small amounts of tissue are needed and these can be of poor quality, such as scales, fins and preserved or archival specimens (Palsbøll, 1999). The initial isolation of the microsatellites is the most time-consuming aspect of this method, but applying identical primers in related species has had some success (Smith *et al.*, 1998; Queller *et al.*, 1993). In addition to the properties of high polymorphism, microsatellites are also able to characterize alleles through differences in the sizes of PCR products. The number of alleles in each microsatellite is very variable and offers the opportunity to choose a marker with the suitable number of alleles at each locus for the particular application in mind (Wright and Bentzen, 1995). Inheritance of microsatellites follows a co-dominant Mendelian fashions and is, therefore, suited to pedigree studies where information on conformation to Hardy-Weinberg expectations needs to be obtained. The versatility of microsatellite markers has made them suitable for application to many aspects of aquacultural genetics. These include stock identification (Noriss *et al.*, 1999; Carlsson *et al.*, 1999), parental assignment (Knight *et al.*, 1998; O’Reilly *et al.*, 1998; Estoup *et al.*, 1998), assessing genetic manipulations and inbreeding (Chapman *et al.*, 1999) and genome mapping (Kocher *et al.*, 1998). The use of microsatellites in selective breeding programs is described below.

### 5. Applications of Microsatellite Markers

Microsatellite DNA consists of small repeat unit, generally less than four nucleotides that generate repeating regions 100-250 bp. These regions are highly interspersed throughout eukaryotic genomes suggested to have a microsatellite sequence distributed once every 10 Kb.

The PCR based microsatellite markers falls in to two groups based on primer design:

1. Those based on arbitrary primers.

2. Those PCR primers designed from a known sequence also known as sequence tagged sites (STS).

The microsatellite generated from arbitrary primers are based on RAPD fragments (randomly amplified microsatellites) is comparatively fast, requires no prior information and circumvents the nontrivial procedure of constructing and screening genomic libraries (Ender *et al.*, 1996; Schierwater *et al.*, 1997). RAPD fragments replace the genomic libraries, and microsatellite loci are detected from RAPD profiles by Southern hybridization with labeled repeats.

STS markers are generated by genomic libraries and detect co-dominant alleles at a single locus or site in the genome. The marker type is very informative in detecting high heterozygosity (69%-88%) within the individual cultivars and high genetic variation between cultivars, making it a useful marker system for plant genome mapping and genome typing. The most informative or polymorphic STS marker appears to be one that amplifies a DNA region containing a microsatellite repeat sequence. Such an STS based marker has been referred to as a simple sequence length polymorphism (SSLP) or Sequence tagged microsatellite site (STMS).

### DNA Fingerprinting and variety identification:

PCR amplified microsatellites are very powerful markers, because they are locus specific, co-dominant, occurs in large number and allow the unambiguous identification of the alleles and data (i.e. primer sequences) can be easily distributed and reproduced among different laboratories. The potential of STMS markers for genotype identification can be illustrated by study on soybean; most of the 96 different genotypes investigated could be discriminated by a set of 7 loci exhibiting 11-26 alleles per locus (Rongwen *et al*., 1995). It was hypothesized that 10 or 15 loci may be adequate to distinguish closely related genotypes, and unique DNA profiles suitable for variety identification. These markers are very useful in gene tagging. The co-dominant nature of the marker helps in the heterozygous allele identification and tagging gene of interest.

### Genetic map construction:

First locus specific microsatellite analysis in plants was published by Akkaya *et al*. (1992). The authors analyzed GenBank derived AT- and ATT- repeats from soybean and found between 6 and 8 alleles depending on the locus. An advantage of STS type markers is the co dominant mode of inheritance permitting easy transfer of markers between genetic maps of different crosses in contrast to the dominant PCR markers type based on arbitrary, which requires the generation of a new map for each cross. The huge number of polymorphic loci combined with the even dispersal of microsatellites throughout the genome makes the STMS analysis a particularly efficient tool for genetic mapping

### Population genetic analysis

Isoenzymes provided population geneticist with discrete co-dominant information. These properties were essential for the development of formulae used to describe the genetic structure of a population. Like isoenzymes, microsatellite have discrete, co-dominant alleles, but they have more alleles and are not affected by the environmental pressure. Microsatellite can therefore be regarded as second generation of population genetic markers.

### Microsatellites and conservation genetics:

Low levels of genetic variation often hamper studies of endangered species. To assess the severity of bottleneck in endangered populations a highly polymorphic class of markers is needed. Microsatellites were found to be informative in several species, which showed almost no variation at other genetic markers (Taylor *et al.,* 1994).

### Cross species amplification of microsatellites:

A major obstacle for microsatellite analysis in the past was the need to develop PCR primers for every species. The demonstration that microsatellite and their flanking regions are conserved across species significantly promoted the widespread use of microsatellites (Schlöterer *et al*., 1991).

### Application in behavioral ecology

#### 1. Male mating success is determined by paternity testing:

Until recently, only field observations were available for estimating success. The limits of this approach are obvious and it is no surprise that genetic analysis of offspring often reveals differences from the predictions of field observation data (Amos *et al*., 1993). Microsatellite are good tool for paternity analysis, as they show co-dominance of discrete alleles. Simple allelic subtraction allows the precise determination of parental inputs, since the offspring inherit one allele from each parent. By analyzing several loci in this way enough information about the father can be collected to identify him. Microsatellite alleles are classified by the length of PCR product and the accurate length determination on a denaturing sequencing gel makes in-gel comparison easy and reliable. This means that once typed an individual can be compared with any other individual just by referring to the determined length of the alleles. This attribute of the microsatellites makes it possible to analyze populations that would otherwise not have been accessible.

2. Social organization of population:

Social insects have been the focus of many studies investigating the basis of sociality. The haplo-diploid genetic system in Hymenoptera provides an experimental setup in which an asymmetry of relatedness is provided: workers in eusocial colonies are unequally related to the male and female of the reproductive provision. Hence, in species where workers control sex allocation a strong bias towards the female is expected as long as colonies that contain single once mated Queen. If the queen is multiple mated or several queen are present in the colony this bias should be less extreme. These theoretical predictions have been tested in the polygamous ants *Myrmica tathensis* (Evans, 1995). Using microsatellites to determine the genetic relationships of colonies, Evans showed that colonies producing male and female sexual often had low levels of relatedness among nest mates. Colonies that produced mainly female sexual were composed of highly related females (workers and female sexual).

Microsatellite based DNA typing is a valuable tool for studying natural populations. For paternity assignment, assessment of genetic relatedness and variety identification, microsatellites are unquestionably the marker of choice. New screening methods, such as capillary electrophoresis, will enhance rapid throughput of large sample sizes for many loci. The high potential for full automation of microsatellite analysis will allow routine typing to occur, which will produce the required data at low cost.

## 6. Microsatellite markers in selective breeding

Genetic markers have a range of uses in selective breeding and broodstock management. The ability to monitor and evaluate populations by examination of the genotype is a great advantage in any program aimed at genetic improvement. The use of PCR techniques in microsatellite analysis means that data can be obtained from all life stages including eggs and larvae, which would have previously been inaccessible to genetic testing. Large numbers of samples can also be rapidly analyzed to produce data within the time-scales of the farming system.

Parental assignment in cultured stocks is one of the primary applications of microsatellites to a breeding program. Microsatellites can identify the parents of high-performing progeny in communal rearing environments and, therefore, assist in the selection process (Garcia de Leon *et al.*, 1998). Family selection becomes possible by collecting genotype information on all the candidates and using family relationships as part of the selection criteria. Walkback-selection (Doyle and Herbinger, 1995) is an example of such a method, whereby fish are selected on size and then on family relationships following microsatellite analysis.

Parental assignment can also be used to reveal information on the contribution of individuals in mass spawning species (Waldbieser and Wolters, 1999; Magoulas, 1998) which can subsequently aid broodstock management. The ability to identify families and individuals allows closer monitoring of stocks within a program and also facilitates communal rearing methods to reduce environmental variance. Inbreeding can severely affect selection efforts and needs to be monitored, in all selection programs. Rates of inbreeding can be estimated from pedigree information and changes in microsatellite allele frequencies over time (Tessier *et al.*, 1997). Inbreeding can then be minimized by the prior identification of the families and individuals that should be used for future breeding (Ryman and Laikre, 1991).

Discriminating between stocks and groups of crocodile can be important to a breeding program where crocodiles are released for restocking natural populations.

Microsatellite tagging can indicate whether performance of cultured crocodile is different to that of wild crocodile in the same habitat (Davis *et al.*, 2001; FizSimmons *et al.*, 2001). Genetic markers are also able to discriminate between farm escapees and wild crocodile (Davis *et al.*, 2001; FizSimmons *et al.*, 2001; Isberg *et al.*, 2003) which may be particularly important for farms undertaking genetic manipulations. Microsatellites have a central role in genome mapping, as they serve as marker points distributed throughout the genome (Magoulas *et al.*, 1998). The production of a physical map of the genome allows the classification of genes affecting commercially important traits (Simm, 1998). In some cases the genes may control an observable phenotype, although regions that contribute a proportion of variation in a quantitative trait can also be determined (Simm, 1998). These regions are known as quantitative trait loci (QTL) and are particularly useful for genetic improvement programmes as complementary to breeding value estimates of genetic merit (Davis and Hertzel, 2000). Many of the traits of interest in a selective breeding programme are quantitative in nature and therefore controlled by many genes of small additive effects. Identifying markers linked to quantitative trait loci can greatly increase the rate of genetic improvement by permitting marker-assisted selection (MAS) (Poompuang and Hellerman, 1997).

Knowledge of quantitative trait loci has several applications for selective breeding in crocodile, as they allow selection of traits that may be difficult or expensive to measure and that can only be measured on one sex. In addition, traits that can only be measured after death or after selection has taken place, such as disease resistance (Davis and Hertzel, 2000) and life time reproductive performance (Simm, 1998), can also be selected. Detection of quantitative trait loci in crocodile species is still quite limited, but studies have been successful in locating QTL's in rainbow trout for spawning time (Sakamoto *et al.*, 1999) and temperature tolerance (Jackson *et al.*, 1998; Danzmann *et al.*, 1999). An increasing number of crocodile species are being mapped, including commercial species, such as *C. porosus*, *A. mississippiensis*, *C. johnstoni* and *C. c. crocodilus*, which raises the prospect of many more QTL sites being available in the future.

Although microsatellites occur in all eukaryotes (Tautz and SchlÖtterer, 1994), their distribution and overall frequency varies widely (Langercrantz *et al*., 1993). Because primer development can be time-consuming and costly, many investigators attempt to use primers developed in one species to amplify orthologous loci in closely related species. Some studies show that this strategy can be quite successful, even in distantly related species. Most such cross-species comparisons, however, have tested either a small number of primer pairs (FitzSimmons *et al*., 1995) or a small number of taxa. Additionally, only loci that are polymorphic in the species from which they are developed are used in most studies. In general, primers that exhibit low levels of variation, as well as those that yield complex or unclear patterns, are discarded without much investigation or hesitation. Thus, it is not known how a reasonably large random sample of microsatellite sequences evolves within a group of taxa.

Gaining an understanding of the genetic population structure of crocodilians and the complexities of their mating systems will contribute to the conservation and management of these species as well as addressing some persistent questions. In the most recent IUCN Action Plan for crocodiles (Ross, 1998), for several species mention is made of the need for information on genetic diversity and relatedness. For example, population genetic surveys are needed of the critically endangered Orinoco crocodile *(C. intermedius),* to assess levels of genetic diversity to inform restocking and reintroduction programs.

FitzSimmons *et al*. (2001) studied on the development and testing of 26 new microsatellite loci for *Crocodylus* spp, which represent the first microsatellite loci found in *Crocodylus.* Microsatellite loci were identified from three species of crocodiles, *C. acutus, C. porosus* and *C. johnstoni* by establishing and screening genomic DNA libraries. DNA was extracted from tail scute or muscle samples following standard phenol/chloroform methods and digested with a combination of *Alul, Haelli, & RsaI.* Digested DNA was run on a 1.2% agarose gel and the 350-600 bp size fragments selected and purified using a QIAquick kit (QIAGEN). Crocodile DNA was blunt-end ligated into pUC18 Smal/BAP plasmids and transformed into fresh competent *E. coli* cells by heat shocking or electroporation. Colonies were grown on Hybond N+ membranes (Amersham, Sydney) and duplicate transferred to Zeta-Probe blotting membranes for hybridization. A synthetic oligonucleotide microsatellite probe (dA-dC)n-(dG-dT)n (Pharmacia) was radioactively labelled by random-priming (Feinberg and Vogelstein, 1983) using a Mega-Prime kit (Amersham) and $\alpha^{32}$P CTP. Filters were hybridized overnight at $65^{0}$ C and the final wash was done in 0.2x SSC and 0.1% SDS at $65^{o}$C. Filters were exposed to X-ray film and positive colonies were selected from the original Hybond colonies after alignment to autoradiography images. This library of potentially positive colonies was subjected to a second round of hybridization to confirm positive status, and the ligated target-plasmid DNA was isolated by a miniprep method (Sambrook *et al.,* 1989) and screened for microsatellites by PCR cycle sequencing with a primer end-labelled with $\gamma^{33}$P. Primers were designed for microsatellite loci containing at least 10 uninterrupted dinucleotide repeats, using OLIGO 4.0-s software. PCR conditions were optimized for all loci and samples from different populations were screened for polymorphism using $\gamma^{33}$p end-labelled primer in the PCR reaction. Products were run on 6% denaturing sequence gels with a sequenced size standard and results were visualized by autoradiography.

The results showed that some microsatellite locus has a large shift in allele size between species, as in the case of *C. porosus* and *C. johnstoni*. Such variation in allele size or in the number of alleles, suggests relatively dynamic loci that may be of interest in the study of microsatellite evolution. Forensic identification can also take advantage of unique allele sizes, or ranges as observed among species, particularly if a multi-locus analysis is undertaken on the specimens of interest. These genetic markers will offer the most powerful insights when they are combined with demographic or behavioural data when available. Additionally, a combined approach using both mtDNA and microsatellite markers will be the most effective for elucidating questions of gene flow and population history.

Glenn *et al.* (1998) revealed that microsatellite loci from American alligator display high levels of intra- and interpopulation variability, demonstrating their utility for studies of alligators. Many of the loci are conserved across crocodilian species, suggesting that some of the loci will be useful as genetic markers in other crocodilians. The microsatellite loci of American alligators were identified from small insert DNA libraries. The PCR primers also produced single amplicons from species of Alligatoridae, Gavialidae, and Crocodilidae, indicating that they may be useful for genetic studies in other crocodilians.

Davis *et al*. (2000) used high degree of polymorphism microsatellite locus that described by Glenn *et al.* (1998) to discover which nests showed evidence of multiple paternity. These genetic data support the longstanding that male alligators successfully mate with more than one female and that females can produce clutches fathered by more than one male. This is a much higher incidence of multiple paternity than found by FitzSimmons (1998) in green sea turtles.

Isberg *et al*. (2004) used microsatellite markers for crocodiles (FitzSimmons *et al*., 2001) to evaluate in farmed saltwater crocodiles for use in parentage testing. The markers evaluated provide an indispensable complement to a selection program for farmed saltwater crocodiles, enabling confirmation of the pedigree records of juveniles from pens of known parents. It is therefore recommended that more microsatellite markers be used in these situations. The use of these microsatellite markers will broaden the scope of a breeding program, allowing progeny to be tested from adults maintained in large breeding lagoons for selection as future breeding animals.

In addition to developing these primers, FitzSimmons *et al*. (2001) evaluated some of the markers on *C. porosus*, although not with the objective of developing a parentage determination kit. Some of these primers have since been used to identify purebred Siamese crocodiles (*C. siamensis*) for reintroduction into Vietnam (FitzSimmons *et al*., 2002).

There has long been an interest in understanding the complexities of crocodile mating systems, given the expectation of alpha male dominance and observed variations in mating behavior (Ferguson, 1985; Vliet, 1989; Kofron, 1990). Do alpha males have the highest reproductive success? What is the variation in male reproductive success and how is this influenced by density and sex ratios? Is there multiple paternity of clutches, and if so, how prevalent is it? To what extent does sperm storage (Ferguson, 1985) and sperm competition influence paternity and hatching success? What are the relationships between mating system, alpha male dominance and migratory behavior? Genetic markers have great potential for testing hypotheses about crocodilian biology built upon data from behavioral and mark-recapture studies.

## 7. Reintroduction of the siamese crocodile

The Crocodile Management Association of Thailand (CMAT) launched its strategy for re-introducing Siamese crocodiles into wild habitats in hopes of partial restoration of these crocodiles in their historical range in Thailand. We conceive that appropriate wildlife conservation comprises not only of preserving species, but also helping animals exist within their own natural habitats.

Crocodilians around the world have been threatened by unavoidable human activities. To alleviate the situation, several conservation and management programs were launched and proved successful in many countries. Unfortunately, not all species receive the same treatment. The Siamese crocodile (*C. siamensis*) in Thailand is the most critical species in term of need for conservation. It has been considered an endangered species based on the small number of specimens remaining in the wild. In 1996 IUCN Red List, *C. siamensis* is categorized as CR: Critically Endangered, Criteria A.1.a. and c. severe decline in numbers and areas more than 80% decline in three generations (Ross, 1998). It is also included in Appendix I of CITES. They appeared to be reduced to non-breeding remnants in marginal habitats. The principal threats are habitat destruction, illegal hunting, and killing as vermin. During a survey in November 1993, Ratanakorn *et al.* (1994) confirmed the presence of at least one wild adult *C. siamensis* in Pang Sida National Park and another in Ang Lue Nai Wildlife Sanctuary. Although wild populations are scarce, *C. siamensis* is abundant in captivity. Tens of thousands of captive populations of pure *C. siamensis* provide a significant resource for restoration.

To return crocodiles into their historic and protected habitats is a major step toward conservation of species. The long term goal is to establish a viable, free-ranging population of Siamese crocodiles in the wild of Thailand, which requires a multidisciplinary approach involving a team of persons drawn from a variety of backgrounds. It also requires an understanding of the effect of the re-introduced species that will have on the ecosystem in order to ascertain the success of the re-introduced population. In addition, a model must be studied for building-up the released population under diverse sets of conditions, in order to specify the optimal number and composition of individuals to be released per year and the numbers of years necessary to ensure establishment of viable population. Positive public relations derived from commercial participation in the program will strengthen perceptions of Thailand's active conservation action and therefore reduce international criticism that can result in inhibitions to trade. The CITES regularization of international trade achieved by approving registrations greatly promoted the economic incentives on which conservation can be based. Therefore, it is a time for those who benefit from trade to provide a structural and policy platform for restoration.

Many conservation and management programs could be applied to conserve *C. siamensis*. Why reintroduction? The IUCN/SSC Reintroduction Specialist Group as an attempt to establish a species in an area which was once part of its historical range, but from which it has been extirpated or become extinct defined reintroduction. The reintroduction of species has been used successfully in many animals and plants, including some crocodilians such as a mugger crocodile (*C. palustris*). Other alternative conservation actions are inappropriate, for example, additional surveys to locate wild animals, enhancement of wildlife protection laws, reduction of illegal hunting, banning of imports and exports, and improvement of protection in national parks. All of these actions are either time-consuming or unnecessary. Moreover, they do not address the problem.

The ideal habitat proposed for restocking of endangered species ought to be both historic and protected areas. A number of species have been bred in captivity to provide a basis for recovery. However, the quality of animals is disputable, especially hybrids and their descendants. Recently, there is a concern about the genetic integrity of released animals. No genetic analysis exists for any of the captive populations; the degree of heterozygosity within populations and the degree of relatedness between isolated populations are completely unknown.

The best approach to the problem is to compare morphological characteristics of both populations, such as skull dimension, body measurement, and other indications. Another alternative approach would be to conduct a microsatellite analysis of DNA samples from a number of participating crocodile farms in Thailand, and compare results with known pure samples.

# MATERIALS AND METHODS

## Materials

### 1. Chemicals

| | |
|---|---|
| Agar: | Fisher # A360-500 |
| Agarose: | Ameresco # 0710-500; Fisher # BP1356-500 |
| Albumin, Bovine (BSA): | Sigma # A6793 |
| Bacto Tryptone: | DifCo # 0123-05-7 |
| Bacto Yeast Extract: | DifCo # 0127-05-3 |
| Phenol: | Ameresco 0945-400mL; Fisher # BP226-500 |
| Chloroform: | Fisher C298-500 |
| Isoamyl Alcohol: | Fisher A393-500 |
| SDS (Laurel Sulfate): | Sigma L4509 |
| EDTA: | Fisher S311-500 |
| dNTP’s: Pharmacia dATP | |
| dCTP | |
| dGTP | |
| dTTP # 27-1890-01 | |
| dA,C,G,TTP | Amresco# |
| Tris: Trizma Base | Sigma T1503 or T8524 |
| Trizma HCl | Sigma T3253 |
| Sigma 7-9 | Sigma T1378 |
| Acrylamide: | BioRad # 161-0123 |
| TEMED: | Sigma # T8133 |
| Ammonium PerSulfate: | Kodak IB70080 |
| X-Gal: | USB # 12385; Diagnostic Chem. Ltd. # 1161 |
| IPTG: | Sigma # I5502; USB # 17884 |
| Ampicillin: | Sigma # A9518 |
| Glycerol: | Sigma G7757 |
| DMSO: | Sigma D5879; Fisher BP231-1 |

## 2.. Enzyme materials

| | |
|---|---|
| *BamH* I: | NEB # 136s |
| *Taq* I : | Promega #R6151 |
| *Cla* I: | NEB # 0197L |
| Taq DNA Polymerase, recombinant: | Life Technology #11615-010 |
| pBluescript II KS+Phagemid Vectors: | Stratagene #212207 |
| XL1-Blue: | Stratagene # 200301 |

## 3. Materials

| | |
|---|---|
| Culture Tubes: | Fisher # 14-961-27 |
| Cuvettes for Gene Pulser: | Stratagene # 165-2089 |
| Filters: NitroBind, Nitrocellulose, 85 mm, | MSI # EP4HY08550 |
| Pasteur Pippettes: | Fisher # 13-678-20B |
| PCR Tubes: 0.5 ml | Midwest Scientific # TC5 |
| Petri Plates: 100 x 15 mm | Fisher # 8-757-13 |
| Pipette Tips: 0-200 µl | |
| 0-1000 µl | |
| 0-50 µl Aeroseal | USA Scientific # 1023-4810 |
| Gene-Images random primer labelling kit | (Amersham Pharmacia Biotech) |
| Hybond nylon membranes | (Amersham, Sydney) |

Kits for non-radioactive colony screening.

Genius 6 Oligonucleotide Tailing Kit - Beheringer Manheim

# Methods

## 1. To construct genomic libraries and primer design for freshwater crocodiles

### 1.1. Blood samples collection

Crocodile blood samples were collected from a wild caught crocodile (*C. siamensis*) from Watsinght Crocodile farm without injury to individual. A self-locking cable snare (Figure 4) captured crocodile. The snare was placed over the crocodiles's head. It was then pulled to an elevated, smooth location and a second snare was used to secure the jaws. A 5-10 ml blood sample was collected in a heparinized syringe from the anterior dorsal sinus and kept on ice until returned to the laboratory.

**Figure 4** Crocodile capturing : the snare was placed over the crocodiles's head (A). Crocodile blood collection: 5-10 ml blood sample was collected in a heparinized syringe from the anterior dorsal sinus (B).

### 1. 2 DNA Extraction

Genomic DNA was isolated from blood sample by standard Proteinase K digestion, followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (Sambrook *et al*., 1989). Determinations of the DNA concentration were assayed by ultraviolet absorption and electrophoresis through agarose gels staining with ethidium bromide (Sambrook *et al.*, 1989).

### 1.3 Clone genomic libraries

Genomic DNA was digested with *Taq* I, in a final volume of 100 µl that composed of 10 µl of 10x *Taq* I buffer, 0.1 U/µl BSA, 0.25 U/µl of *Taq* I, and 1 µg/µl of genomic DNA. The mixture was incubated at 65°C for at least five hours or overnight. Digested DNA was run on a 0.8% agarose gel with ethidium bromide and visualized under UV. DNA fragments with an average size of 500 to 1000 bps were isolated from agarose gel and purified by using QIAquick spin column (QIAGEN) followed by ethanol precipitation. This isolated DNA fragments were ligated into the *Cla* I site pBluescript II KS+ phagemid (Stratagene) and transformed into fresh competent XL1-Blue supercompletent cells (Stratagene) by heat shocking. Transformed cells were grown up overnight on LB agar plates containing 50 µg/ml ampicillin with X-Gal and IPTG. Recombinant colonies were transferred onto Hybond nylon membranes (Amersham, Sydney) and followed by hybridizing with synthetic oligonucleotide microsatellite probe $d(AC)_{15}$. Prehybridization and hybridization were carried out at 42°C in 6x SSC (from a 20x stock = 3M NaCl, 0.3 M sodium citrate), 5x denhardt reagent (from a 50x stock = 1% BSA fraction V, 1% Ficoll and 1% polyvinylpropylene) and 0.1% SDS. After hybridization, it was washed twice (30 min) in a 6x SSC, 0.1% SDS solution. Filters were screened for microsatellite repeated by using Gene-Images random primer labelling kit (Amersham Pharmacia Biotech) and exposed to X-ray film. Positive colonies were selected from the original Hybond colonies after alignment to autoradiography images and a recombinant plasmid were isolated by using alkaline preparation and screened for recombinants DNA by restriction enzyme. The potentially positive recombinants were sequenced automatically (Fluorescent dye method, Applied Biosystems).

### 1.4 Primer design

Primer pairs were designed to complement the flanking region of microsatellite and its should have the following properties: (1) lack of tandem repeats and a distance of 10-60 base pairs before microsatellite repeat in both directions; (2) size of 18-24 bases with 50-65% of G and C; (3) annealing temperature between 50-

65°C with a maximum 4°C difference between the two primers of any single pair; (4) no primer dimer formation, either with itself or any other member of the pair by using Genetyx software (GENETYX software development Co. Ltd., Tokyo, Japan). The new microsatellite primers were named according to the scientific name of the organism, using the first letter of the genus and species in capitals and followed by the number representing the crescent numerical order. For example, the first new microsatellite marker was named as *CS*-1 (*CS*= *Crocodylus siamensis*).

## 2. To test the primers that had been designed

Five individuals of *C. siamensis* from Sriracha breeding farm were used to test and analyze the microsatellite designed primer. Blood was collected by puncturing the anterior dorsal sinus which runs posteriorly through the skull of crocodile. DNA from these samples was purified by phenol/chloroform extraction followed by isopropyl alcohol precipitation (Sambrook *et al.*, 1989). The samples were amplified in a 25 µl final volume of 1x PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), the appropriate mM $MgCl_2$ , 0.2 mM each dNTP, 0.4 mM of each primer, 0.02 U/µl. Taq DNA polymerase, and 25 ng of DNA. The amplified conditions are started with denaturing step of 94°C for 3 min and followed by 35 cycles of 94°C for 1 min, the appropriate annealing temperature for 1 min, and amplification at 72°C for 1 min. Products were stored at 4°C until ready to be analyzed and scored. The alleles of the microsatellite primers were detected in 6% denaturing polyacrylamide gel electrophoresis and their expected sizes were compared with standard size of ϕx*Hinf*I. The program Kodak 1D Digital Science V. 3.0.2: Scientific Imaging System (Eastman Kodak Company, New Haven, CT) was used for analyses of scientific images.

## 3. To determine specific marker for identified freshwater and saltwater crocodiles

### 3.1 Crocodile Blood Samples

Blood samples of 29 freshwater crocodile (*C. siamensis*), 4 saltwater crocodile (*C. porosus*), 5 *Caiman crocodilus crocodilus* and 2 Hybrid (*C. siamensis* x *C. porosus*) were collected from the anterior dorsal sinus. The procedure of blood withdrawal and DNA purification are the same as described earlier.

### 3.2 PCR condition

Microsatellite loci were ampified under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, the appropriate annealing temperature for 1 min, and amplification at 72°C for 1 min. The samples were amplified in a 25 µl final volume of 1x PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), the appropriate mM $MgCl_2$ , 0.2 mM each dNTP, 0.4 mM of each primer, 0.02 U/µl Taq DNA polymerase, and 25 ng of DNA. Products were stored at 4°C until ready to be analyzed and scored. The alleles of the microsatellite markers were detected in 6% denaturing polyacrylamide gel electrophoresis and their sizes were measured by the use of the program Kodak 1D Digital Science V. 3.0.2: Scientific Imaging System (Eastman Kodak Company, New Haven, CT), a system for analyses of scientific images, ϕx*Hinf*I fragments as size standards. The observed size range for each locus is defined as the variation from minor to major bands appearing at genotypes of individuals sampled for each marker.

### 3.3 Scoring Microsatellite Loci

The band (alleles) can be scored as presence/absence (1 or 0). Alleles can be coded simply as their integer size in base pair in the case of co-dominant markers which heterozygous yield two band and those that are homozygous yields one band.

### 3.4 Data analysis that can be grouped into two main categories:

### 3.4.1 Allelic frequencies

Allelic frequencies were computed using the following equation (Hedrick, 2000).

$$p = \frac{N_{12} + 2N_{11}}{2N}$$

Where, p = a frequency of an allele A,

$N_{11}$ = a number of AA homozygotes,

$N_{12}$ = a number of heterozygotes having Aa allele,

N = number of individuals

### 3.4.2 Estimation of heterozygosity

3.4.2.1 Observed heterozygosity (Ho) is the average of actual observed heterozygotes at each locus. It is calculated by direct counting the observed heterozygote individuals at each locus and dividing by the sample number.

3.4.2.2 The calculation of expected heterozygosity per individual (he) was performed by the following equation:

$$he = 1 - \frac{\sum xi}{2}$$

Where, he is the expected heterozygosity per individual

Xi = allele frequency of $i^{th}$ allele at a locus in population X

The expected mean heterozygosity per locus (He) is the sum of (he) over loci including monomorphic loci (where he= 0) divided by a total number of loci examined,

$$He = \frac{\sum he}{r}$$

Where, r = total number of locus

The calculation of Ho and He was done by a GENEPOP software package (Raymond and Rousset, 2001)

# RESULTS AND DISCUSSIONS

## 1. To construct genomic libraries

Genomic DNA of a wild *C. siamensis* was isolated from whole blood cells by standard Proteinase K digestion. DNA concentration and purity were assayed by ultraviolet absorption and electrophoresis through 0.8% agarose gels stained with ethidium bromide (Figure 5) assayed DNA concentration and purity. The result showed the total of purified genomic DNA was $\geq$ 1000 ng /μl.

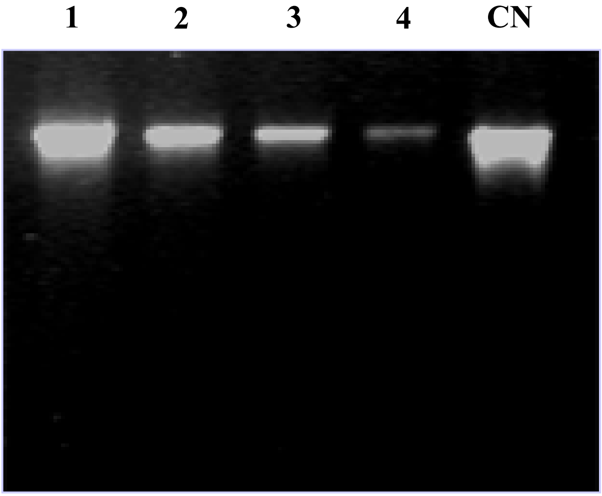

**Figure 5** Agarose gel electrophoresis of genomic DNA purified with phenol/chloroform/isoamyl alcohol extraction.

Lane 1: Lamda DNA 1000 ng /μl
Lane 2: Lamda DNA 500 ng /μl
Lane 3: Lamda DNA 300 ng/μl
Lane 4: Lamda DNA 100 ng /μl
Lane 5: purified DNA isolated from crocodile whole blood (CN)

Genomic DNA was digested with *Taq* I to a final volume of 100 μl. Digested DNA was run on a 0.8% agarose gel stained with ethidium bromide and visualized under UV-light. The result represented that genomic DNA was digested to small fragment varies in size and showed smear in 0.8% agarose gel electrophoresis (Figure 6). DNA fragments between 500 to 1000 bps were selected and isolated from the gel.

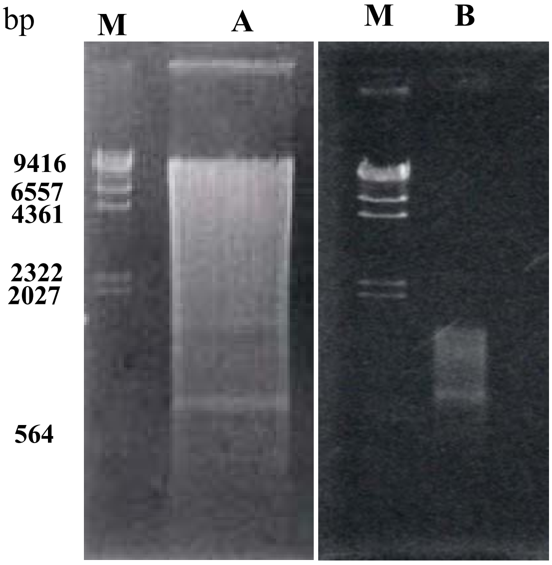

**Figure 6** Agarose gel electrophoresis of genomic DNA digested with *Taq* I restriction endonuclease, run in a 0.8% agarose/TBE gel and stained with ethidium bromide.

M: Lambda *Hin*d III marker

A: Digested DNA showed long smear

B: Approximately 500-1000 bps of purified DNA fragments

Selected DNA fragments were purified using QIAquick spin column followed by ethanol precipitation. They were run on a 0.8% agarose gel stained with ethidium bromide and visuallized under UV-light (Figure 6). Digested DNA fragments were stricky-end ligated into pBluescript II KS+ phagemids and transformed into fresh competent XL1-Blue *E. coli* host cells (Stratagene) by heat shocking. They were plated on standard LB-ampicillin plates that was the selective media containing 100 μ g/ml ampicilin, 0.5 mM IPTG and 80 μg/ml X-Gal and grown overnight. The positive clone was checked by white-blue colony screening (Figure 7).

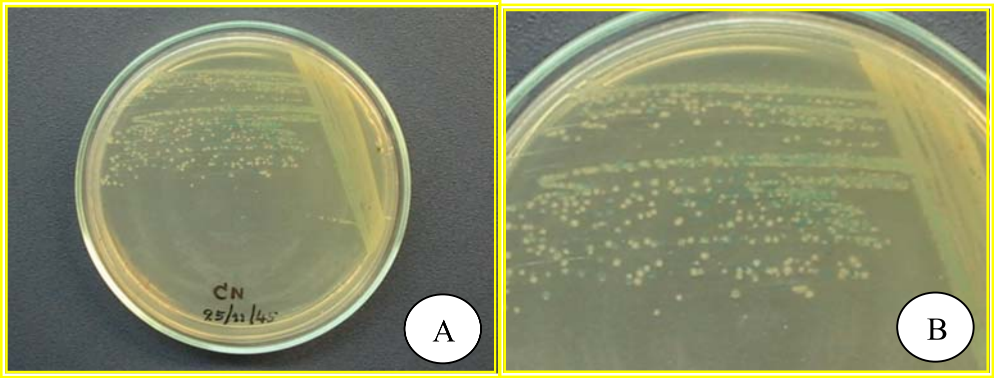

**Figure 7** LB-ampicillin plates showed white-blue colonies after incubated for overnight. (A) The whole plate. (B) The colonies in high magnification.

Total 215 clones of transformant were constructed and hybridized with d(AC)$_{15}$ probe. Filters were exposed to X-ray film. The result showed positive clones in deep black color (Figure 8).

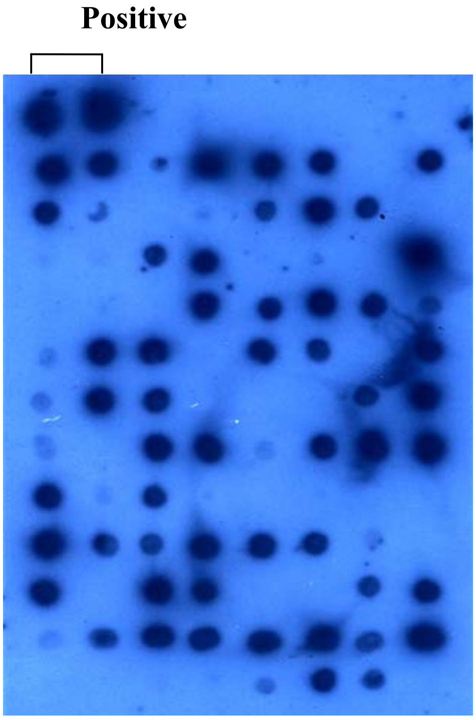

**Figure 8** Detection of microsatellite DNA on X-ray film. Positive recombinants showed deep black in color.

The result demonstrated that 59 clones or 27.44% of total clones with tandem repeat nucleotide had larger size than pBluescript II KS+ phagemids DNA (Figure 9). From these positive clones, 30 of them (50.7% of total $d(AC)_{15}$ positive clones) were selected for DNA sequencing. The other twenty-nine clones can not be studied further due to inadequate lengths of sequence.

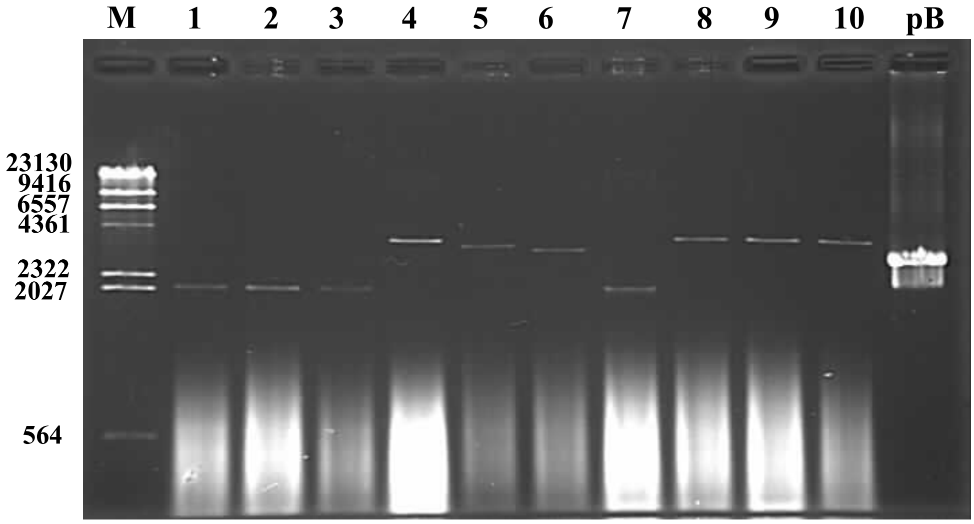

**Figure 9** Restriction patterns of recombinant plasmid DNA digested with *BamH* I.

Lane 1, 2, 3, 7: The tandem repeat nucleotides showed smaller size than pBluescript II KS+ phagemids DNA

Lane 4, 5, 6, 8, 9, 10: The nucleotides showed larger size than pBluescript II KS+ phagemids DNA

M: Lambda *Hin*d III marker

pB: pBluescript II KS+ phagemids DNA

## Primer design and nomenclature of the successive microsatellite locus

The DNA sequencing results gave only 22 clones that can be selected to design primer. The other eight clones can not be studied further due to inadequate lengths of flanking sequence. Thirty-two microsatellite primers were designed from 22 positive clones and only 20 of these provided reliable amplifications and they were tentatively named as *CS*-2 to *CS*-35 (Table 2). The DNA sequences of the cloned alleles were submitted to GenBank (accession numbers **EF413033-EF413054**). The entire nucleotide sequences designated GenBank EF413033-EF413054 is present in the appendix (Table 1-19). The sequencing product of each microsatellite locus can be classified as one of three types according to Weber (1990): (a) perfect sequences without any interruptions in the repeats; (b) imperfect sequences with one or more interruptions in the run of repeats; and (c) compound sequences with a run of CA or GT repeats, adjacent to a block of short tandem repeats of different sequence, usually another type of nucleotide repeat (GA, TC, or AT repeats). Four (20% of total microsatellite primers) of the 20 microsatellite primers reported were compound microsatellites, sixteen (80% of total microsatellite primers) were perfect repeats (Table 2).

**Table 2** Primer parameters and observed of twenty *Crocodylus siamensis* microsatellite loci.

| Locus (GenBank accession no.) | Primer: ( 5' ---> 3' ) | Lenght | GC% | TM | Repeats | Expected size (bp) | PCR Condition | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Annealing (°C) | Mg (mM) | Cycle |
| *CS*-2 | F: GCAAACCTATCACCAGTTCAAGC | 23 | 47.82 | 58.76 | $(CA_8)$ | 230 | 68 | 1.5 | 40 |
| (EF4130039) | R: TCTCTGTCACCTGAACCTTCCTC | 23 | 52.17 | 58.80 | | | | | |
| *CS*-4 | F: CCATGCCCTACCACACAACTG | 21 | 57.14 | 58.79 | $(AC_{13})$ | 200 | 65 | 1.5 | 35 |
| (EF4130051) | R: CAAACACAAAGGCATTCAAAGATG | 24 | 37.50 | 58.69 | | | | | |
| *CS*-5 | F: TCTCTCCTTCTTTCTGCTTGTGC | 23 | 47.82 | 58.76 | $(AC_{16})$ | 200 | 65 | 1.5 | 35 |
| (EF4130052) | R: GTGCAAGGTCTGTACCTGTGTGA | 23 | 52.17 | 58.80 | | | | | |
| *CS*-10 | F: TGACAGTGGCTTTATTGAACAGG | 23 | 43.47 | 58.72 | $(GC_5AC_{17})$ | 240 | 65 | 1.5 | 30 |
| (EF4130033) | R: TGGACTCTCTCCTCCTGGACTTC | 23 | 56.52 | 58.85 | | | | | |
| *CS*-12 | F: GGACAGCAACAGAAAAGACAGG | 22 | 50.00 | 58.75 | $(AG_8)$ | 200 | 60 | 1.5 | 35 |
| (EF4130034) | R: ATAGGAAGCGTTCGTGCTGATG | 22 | 50.00 | 58.75 | | | | | |
| *CS*-14 | F: CCTTCATGTGGATTAGGAACAGG | 23 | 47.82 | 58.76 | $(AC_{10})$ | 270 | 60 | 1.5 | 35 |
| (EF4130035) | R: TAGCAGCTTGAAGTGGGTAGCAG | 23 | 52.17 | 58.80 | | | | | |
| *CS*-15 | F: TCACCTGCATATTTCTCCTTCCA | 23 | 43.47 | 58.72 | $(TC_6)$ | 220 | 65 | 1.5 | 35 |
| (EF4130036) | R: GACCGGGGCTCAGTAAAACAC | 21 | 57.14 | 58.79 | | | | | |
| *CS*-17 | F: GATCCCTCCTACACACACACG | 21 | 57.14 | 58.79 | $(GC_5AC_{12})$ | 180 | 60 | 1.5 | 35 |
| (EF4130037) | R: TATTAGGGCTGGACAGTCAAAGG | 23 | 47.82 | 58.76 | | | | | |
| *CS*-18 | F: GCAGAACCATAACACATGCACAG | 23 | 47.82 | 58.76 | $(A_{19})$ | 240 | 60 | 1.5 | 35 |
| (EF4130038) | R: GCATTCCATAGTCCCCTCCATAG | 23 | 52.17 | 58.80 | | | | | |
| *CS*-20 | F: CCTTCCGCCCAAAATAATTGC | 21 | 47.61 | 58.69 | $(C_{10}AC_5)$ | 249 | 60 | 1.5 | 35 |
| (EF4130040) | R: CCCTGCACTAACAAGGAGCAG | 21 | 42.85 | 58.79 | | | | | |

**Table 2** (Continued)

| Locus (GenBank accession no.) | Primer: ( 5' ---> 3' ) | Lenght | GC% | TM | Repeats | Expected size (bp) | PCR Condition | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Annealing (°C) | Mg (mM) | Cycle |
| *CS*-21 | F: ATTTCCACTCACAGCTTCAAACC | 23 | 43.47 | 58.72 | $(AC_5)$ | 260 | 60 | 1.5 | 35 |
| (EF4130041) | R: GGATGCTTGTAGGTCTTGTTTAGC | 24 | 45.83 | 58.77 | | | | | |
| *CS*-22 | F: CTGTAGGCTGTGACAAAATCCTG | 23 | 47.82 | 58.76 | $(AC_7)$ | 180 | 60 | 1.5 | 35 |
| (EF4130042) | R: CATAACCAACCAGAATGTGACTGC | 24 | 45.83 | 58.72 | | | | | |
| *CS*-24 | F: GAACCCAGGAGGCAGGAGAG | 20 | 65.00 | 58.83 | $(AC_{26})$ | 270 | 68 | 1.5 | 35 |
| (EF4130043) | R: CACATACACAGAACCCAGGTGTG | 23 | 52.17 | 58.80 | | | | | |
| *CS*-25 | F: ATCCTCAGTGTCATCTGCTCACC | 23 | 52.17 | 58.80 | $(AC_9)$ | 180 | 65 | 1.5 | 35 |
| (EF4130044) | R: TCTTCCCTGCTCACCTTTCTTTC | 23 | 47.82 | 58.76 | | | | | |
| *CS*-26 | F: GCCATGTGTACTAACTGGGAAGTC | 24 | 50.00 | 58.81 | $(AC_{17})$ | 240 | 65 | 1.5 | 35 |
| (EF4130045) | R: GCCATTTTTAGTCAGGTTGTTGC | 23 | 43.47 | 58.72 | | | | | |
| *CS*-28 | F: CAGTCTCCAGCACTTGGGGATAG | 23 | 56.52 | 58.85 | $(AC_{15})$ | 200 | 68 | 1.5 | 35 |
| (EF4130046) | R: TTGGCTACAAAGGACCAACTCAC | 23 | 47.82 | 58.76 | | | | | |
| *CS*-30 | F: ACTCACACTACCATCTCCAGCAC | 23 | 52.17 | 58.80 | $(AC_{15})$ | 210 | 68 | 1.5 | 35 |
| (EF4130047) | R: TGTGTGTCATGTGCGTGAACC | 21 | 52.38 | 58.74 | | | | | |
| *CS*-32 | F: GTACCAAGCCCCTTTAACACCTG | 23 | 52.17 | 58.80 | $(AC_{17})$ | 245 | 68 | 1.5 | 35 |
| (EF4130048) | R: GGGGAGAAGGAAACTAGGAGAGG | 23 | 56.52 | 58.85 | | | | | |
| *CS*-33 | F: ATCAACTTTCAGCCTGGGATAGG | 23 | 47.82 | 58.76 | $(AC_{12})$ | 200 | 68 | 1.5 | 40 |
| (EF4130049) | R: ATGTCTTATTGCTTCCCTTGCAC | 23 | 43.47 | 58.72 | | | | | |
| *CS*-35 | F: GTCTGAAAAGGGTGTTTGTG | 20 | 45.00 | 58.63 | $(AC_{17})$ | 230 | 60 | 105 | 35 |
| (EF4130050) | R: CCTAAACTGTTAGAAGCCAAG | 21 | 42.85 | 58.64 | | | | | |

## 2. Designed primer test

In order to test the polymorphism of 20 successive primers that had been designed, 5 captive freshwater crocodiles were used. The result showed that the allele number of each locus was range 1-4 alleles (Table 3), with an average of only 1.7 alleles per locus. The average length of uninterrupted repeats from the selected clones was 12.3. Ten (50 % of total microsatellite primers) of the 20 microsatellite loci were polymorphic.

The enrichment procedure that we employed was successful. The number of microsatellite library obtained in our study was di-nucleotide repeats which are common in Crocodylidae and Alligatoridae as previously reported by Glenn *et al.* (1998) for *A. mississipiensis*, FitzSimmons *et al.* (2001) for .*C. porosus* and *C. johnstoni*, and Zucoloto *et al.* (2002) for *Caiman latirostris.*

**Table 3** Primer parameters and observed values for 5 freshwater crocodiles (*C. siamensis*).

| Locus<br>(GenBank accession no.) | Primer: ( 5' ---> 3' ) | Expected size (bp) | Observed allele size range (bp) | NA |
|---|---|---|---|---|
| *CS*-2<br>(**EF4130039**) | F: GCAAACCTATCACCAGTTCAAGC<br>R: TCTCTGTCACCTGAACCTTCCTC | 230 | 230-250 | 2 |
| *CS*-4<br>(**EF4130051**) | F: CCATGCCCTACCACACAACTG<br>R: CAAACACAAAGGCATTCAAAGATG | 200 | 200-210 | 2 |
| *CS*-5<br>(**EF4130052**) | F: TCTCTCCTTCTTTCTGCTTGTGC<br>R: GTGCAAGGTCTGTACCTGTGTGA | 200 | 190-210 | 2 |
| *CS*-10<br>(**EF4130033**) | F: TGACAGTGGCTTTATTGAACAGG<br>R: TGGACTCTCTCCTCCTGGACTTC | 240 | 240-250 | 2 |
| *CS*-12<br>(**EF4130034**) | F: GGACAGCAACAGAAAAGACAGG<br>R: ATAGGAAGCGTTCGTGCTGATG | 200 | 200 | 1 |
| *CS*-14<br>(**EF4130035**) | F: CCTTCATGTGGATTAGGAACAGG<br>R: TAGCAGCTTGAAGTGGGTAGCAG | 270 | 270 | 1 |
| *CS*-15<br>(**EF4130036**) | F: TCACCTGCATATTTCTCCTTCCA<br>R: GACCGGGGCTCAGTAAAACAC | 220 | 210-230 | 4 |
| *CS*-17<br>(**EF4130037**) | F: GATCCCTCCTACACACACACG<br>R: TATTAGGGCTGGACAGTCAAAGG | 180 | 170-200 | 2 |
| *CS*-18<br>(**EF4130038**) | F: GCAGAACCATAACACATGCACAG<br>R: GCATTCCATAGTCCCCTCCATAG | 240 | 240 | 1 |
| *CS*-20<br>(**EF4130040**) | F: CCTTCCGCCCAAAATAATTGC<br>R: CCCTGCACTAACAAGGAGCAG | 249 | 249 | 1 |
| *CS*-21<br>(**EF4130041**) | F: ATTTCCACTCACAGCTTCAAACC<br>R: GGATGCTTGTAGGTCTTGTTTAGC | 260 | 260-270 | 2 |
| *CS*-22<br>(**EF4130042**) | F: CTGTAGGCTGTGACAAAATCCTG<br>R: CATAACCAACCAGAATGTGACTGC | 180 | 180 | 1 |
| *CS*-24<br>(**EF4130043**) | F: GAACCCAGGAGGCAGGAGAG<br>R: CACATACACAGAACCCAGGTGTG | 270 | 270 | 1 |
| *CS*-25<br>(**EF4130044**) | F: ATCCTCAGTGTCATCTGCTCACC<br>R: TCTTCCCTGCTCACCTTTCTTTC | 180 | 180 | 1 |
| *CS*-26<br>(**EF4130045**) | F: GCCATGTGTACTAACTGGGAAGTC<br>R: GCCATTTTTAGTCAGGTTGTTGC | 240 | 240 | 1 |
| *CS*-28<br>(**EF4130046**) | F: CAGTCTCCAGCACTTGGGGATAG<br>R: TTGGCTACAAAGGACCAACTCAC | 200 | 180-200 | 2 |
| *CS*-30<br>(**EF4130047**) | F: ACTCACACTACCATCTCCAGCAC<br>R: TGTGTGTCATGTGCGTGAACC | 210 | 210 | 1 |
| *CS*-32<br>(**EF4130048**) | F: GTACCAAGCCCCTTTAACACCTG<br>R: GGGGAGAAGGAAACTAGGAGAGG | 245 | 245 | 1 |
| *CS*-33<br>(**EF4130049**) | F: ATCAACTTTCAGCCTGGGATAGG<br>R: ATGTCTTATTGCTTCCCTTGCAC | 200 | 200-210 | 2 |
| *CS*-35<br>(**EF4130050**) | F: GTCTGAAAAGGGTGTTTGTG<br>R: CCTAAACTGTTAGAAGCCAAG | 230 | 230-240 | 2 |

* NA: total number of alleles.

### 3. To determine specific band for freshwater and saltwater crocodiles

Twenty microsatellite loci were amplified by polymerase chain reaction (PCR) to assess the level of polymorphism in freshwater (*C. siamensis and C. c. crocodilus*) and saltwater crocodile (*C. porosus*). The amplifications were successful across eighteen loci (*CS*-2, *CS*-4*,* *CS*-5, *CS*-10, *CS*-12, *CS*-14, *CS*-15, *CS*-17, *CS*-18, *CS*-20, *CS*-21, *CS*-22, *CS*-24, *CS*-25, *CS*-26, *CS*-30, *CS*-32, and *CS*-35) for the 40 crocodiles but only two loci (*CS*-28, and *CS*-33) were generated unclearly interpretable PCR products.

The PCR conditions used were those optimal for amplification of microsatellite loci from *C. siamensis* and thus may not be optimal in the other species conditions (i.e., the most stringent conditions that yielded products in *C. siamensis*) to test all taxa. Polyacrylamide Gel electrophoresis illustrating characteristic polymorphism in some loci; CS-4, CS-26, CS-25, CS-17, CS-22, CS-35, CS-20, CS-5 and CS-32 are presented in Figure 10-18.

**Figure 10** Microsatellite amplification products from locus *CS*-4 submitted to polyacrylamide gel electrophoresis (PAGE) and visualized by silver nitrate staining. From left to right: lanes 1–6, 9-26, 29 and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s+C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

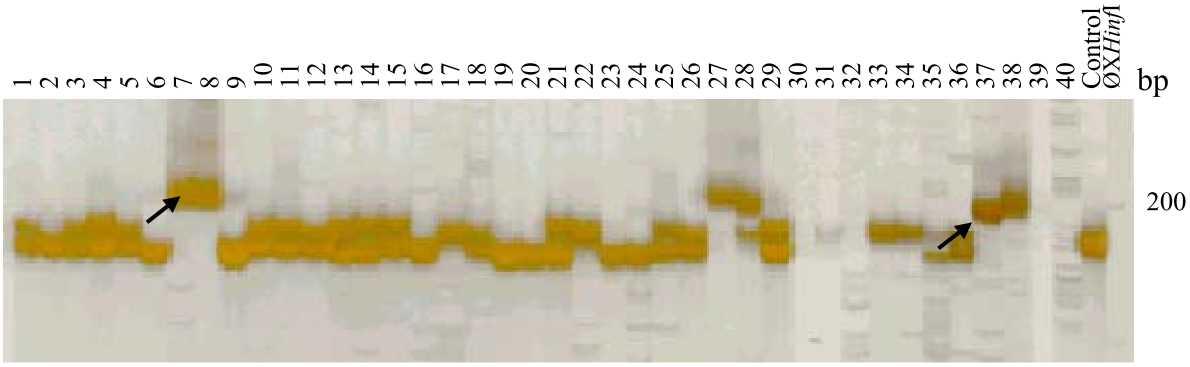

**Figure 11** Microsatellite amplification products from locus *CS*-26 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right lanes 1–6, 9-26, 29 *C. siamensis* and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

PCR products from locus *CS*-4 (Figure 10), *CS*-26 (Figure 11) and *CS*-30 respectively showed a clear pattern of single specific band (arrow) in *C. porosus.* Additionally, all products scored were similar in size to the amplicons from *C. porosus*.

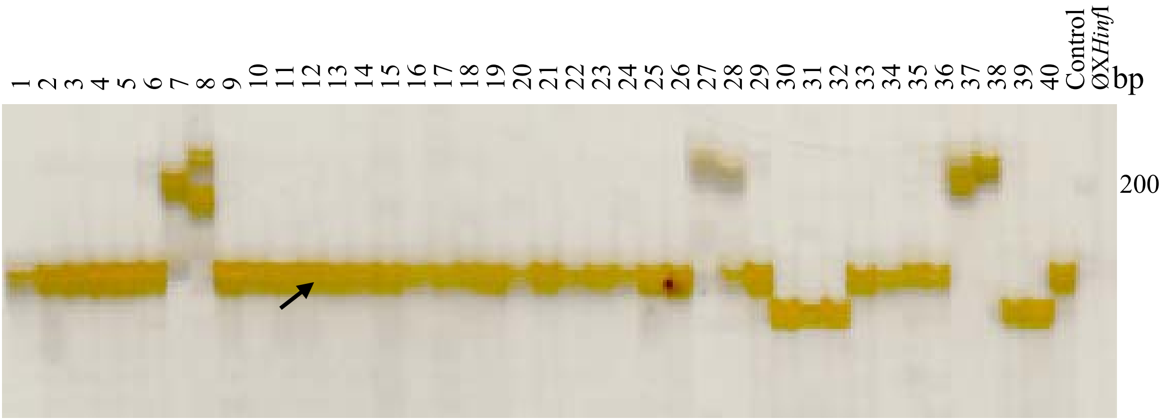

**Figure 12** Microsatellite amplification products from locus *CS*-25 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right: Lanes lanes 1-6, 9-26, 29 *C.* and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

PCR product from locus *CS*-25 (Figure 12) showed a clear pattern of single specific band (arrow) in *C. siamensis*. Additionally, all products scored were similar in size to the amplicons from *C. siamensis*.

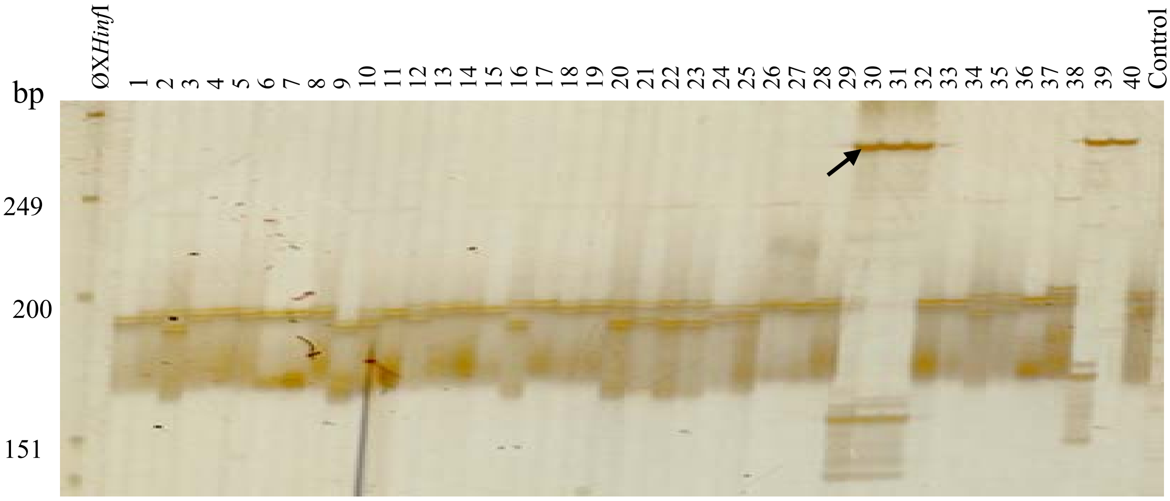

**Figure 13** Microsatellite amplification products from locus *CS*-17 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right, lanes 1–6, 9-26, 29 and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

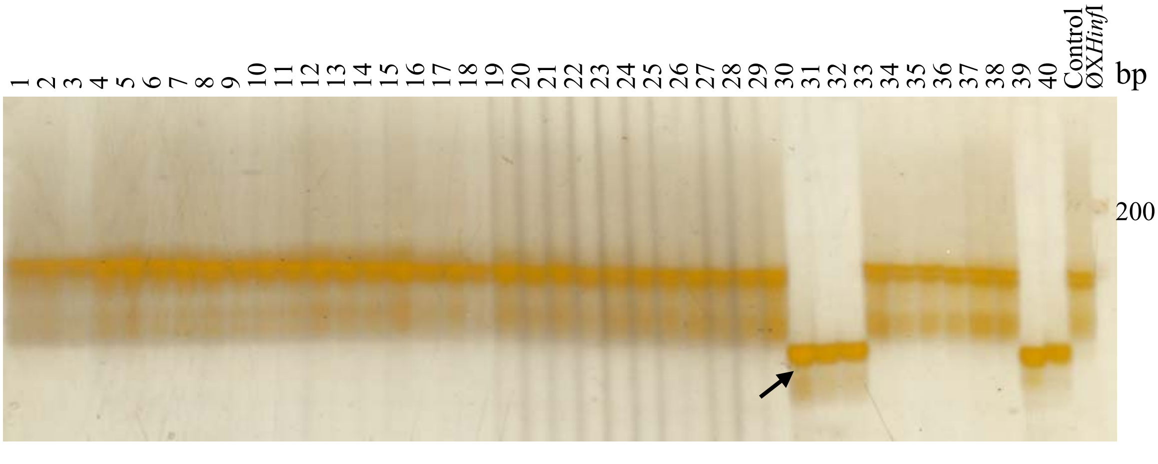

**Figure 14** Microsatellite amplification products from locus *CS*-22 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right lanes 1–6, 9-26, 29 *C.* and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

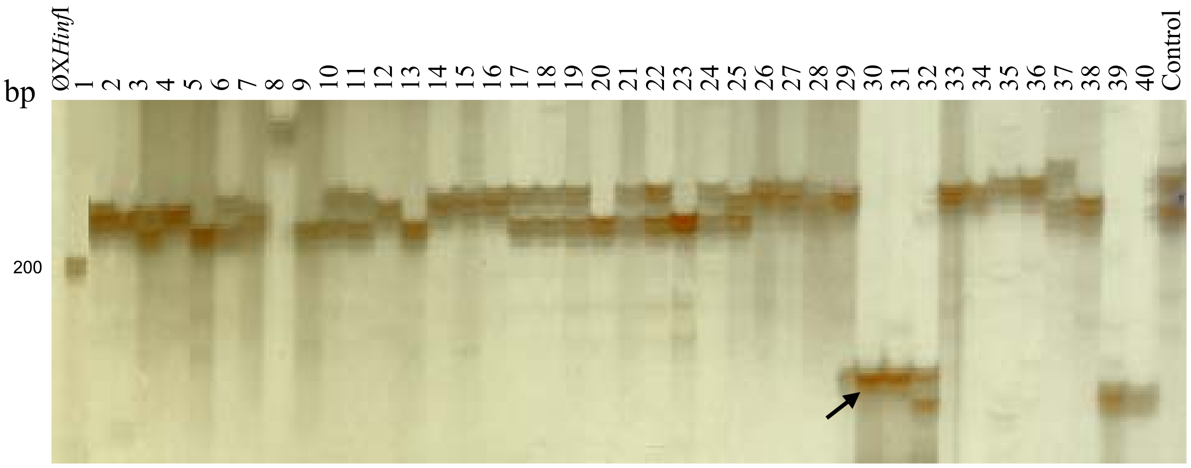

**Figure 15** Microsatellite amplification products from locus *CS*-35 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right lanes 1–6, 9-26, 29 and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s+C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

PCR products from locus *CS*-2, *CS*-7, *CS*-10, *CS*-12, *CS*-14, *CS*-17 (Figure 13), *CS*-22 (Figure 14), *CS*-24 and *CS*-35 (Figure 15) showed a clear pattern of single specific band (arrow) in *C. c. crocodilus.* Additionally, all products scored were similar in size to the amplicons from *C. c. crocodilus.*

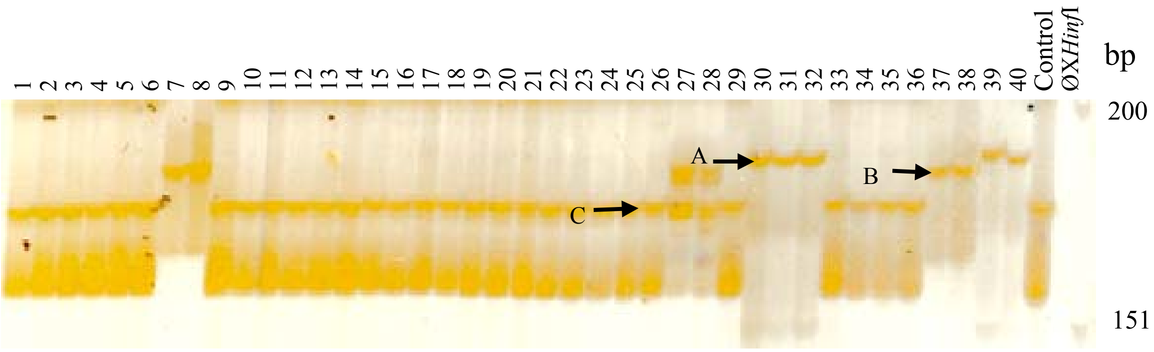

**Figure 16** Microsatellite amplification products from locus *CS*-20 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right: lanes 1–6, 9-26 and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s+C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

PCR product from locus *CS*-20 (Figure 16) showed a clear pattern of single specific band (arrow) in *C. c. crocodilus* (arrow A), in *C. porosus* (arrow B) and in *C. siamensis* (arrow C). Additionally, all products scored were similar in size to the amplicons for each species.

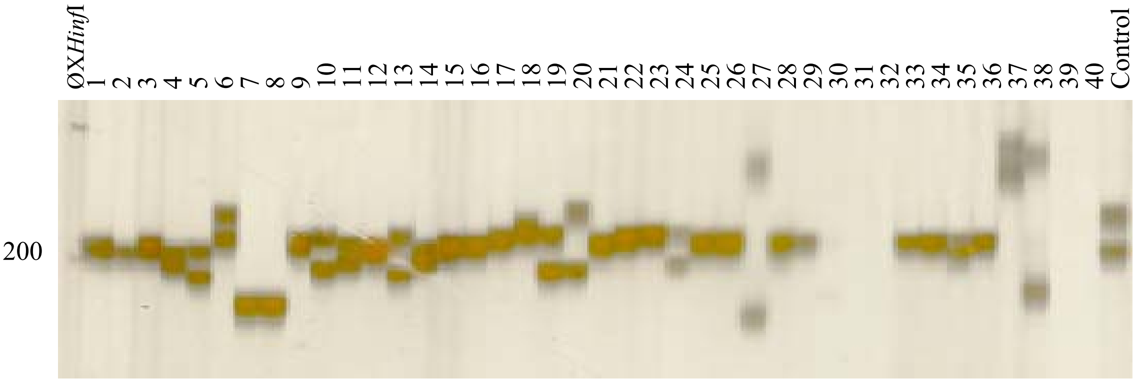

**Figure 17** Microsatellite amplification products from locus *CS*-5 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right lanes 1–6, 9-26, 29 *C. siamensis* and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis.*

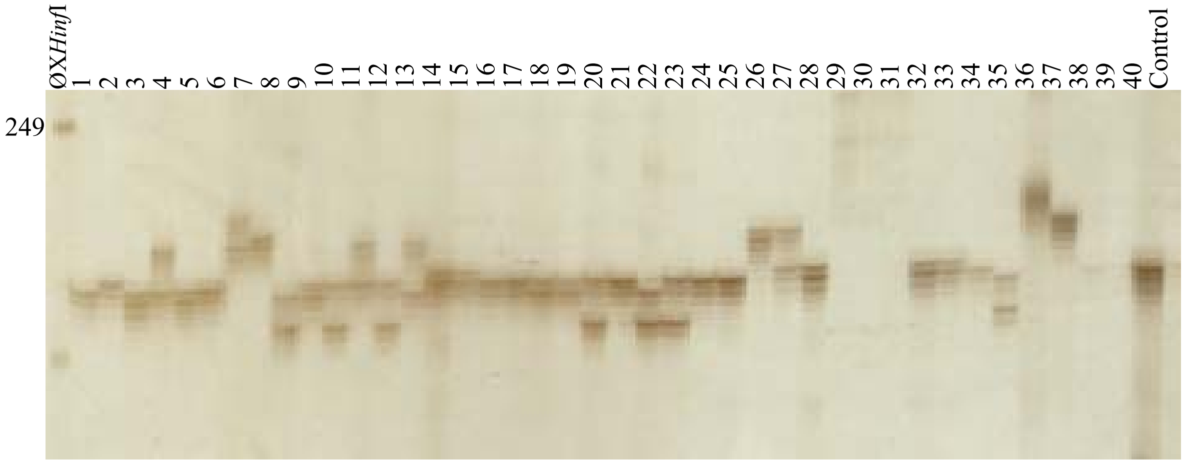

**Figure 18** Microsatellite amplification products from locus *CS*-32 submitted to polyacrylamide gel electrophoresis and visualized by silver nitrate staining. From left to right lanes 1–6, 9-26, 29 *C. siamensis* and 33-36 *C. siamensis*, lanes 27-28 hybrid (*C.s*+*C.p*), lanes 7-8 and 37-38 *C. porosus*, lanes 30-32 and 39-40 *C. c. crocodilus* and control = wild *C. siamensis*.

PCR product from locus CS-4 (Figure 10), *CS*-26 (Figure 11), *CS*-17 (Figure 13), *CS*-35 (Figure 15), *CS*-5 (Figure 17), *CS*-32 (Figure 18), *CS*-10, *CS*-12, *CS*-18, *CS*-21, *CS*-24, and *CS*-30 showed a clear polymorphic pattern in *C. siamensis.* Average observed heterozygosity (Table 3) was 0.37 (range 0.14–0.61) and average expected heterozygosity was 0.41 (range 0.12–0.68). Furthermore, absent alleles were detected for seven loci CS-4 (Figure 10), *CS*-5 (Figure 17), *CS*-18, *CS*-21, *CS*-26 (Figure 11), *CS*-30, and *CS*-32 (Figure 18) in *C. c. crocodilus* individuals.

Even with these results, the initial applications of these primers are promising. These data from populations of *C. siamensis, C. porosus* and *C. c. crocodilus* indicated that several loci would be quite useful in studies of identify species of crocodile. In a captive population of *C. siamensis,* it appears that there will be enough genetic variation to determine paternal exclusion in clutches. It is hoped that these primers will enable the application of genetic investigations in other studies of order Crocodylia. As in other species, these genetic markers will offer the most powerful insights when they are combined with demographic or behavioral data when available. Additionally, a combined approach using both mtDNA and microsatellite markers will be the most effective for elucidating questions of gene flow and population history. It is hope that the availability of these microsatellite markers will allow comparative studies of mating systems and population structure among crocodilians.

For heterozygosity, the observed heterozygosity (Ho) was very low in *C. siamensis* ranging from 0.14-0.61 (Appendix Table 20). This indicated that most of *C. siamensis* have inbred in the same species, or have pure bred in the farm. Thus, selection for individuals with higher heterozygosity as is typically recommended (Moritz, 1999) is possible for the planned reintroduction into National Park. This strategy is indicated theoretically, to provide a greater range of options upon which selection can, if indeed variation at dinucleotide microsatellite loci provides a surrogate measure of variation in coding regions of the genomic DNA. Whether or not this assumption is met, selecting individuals with higher heterozygosity (if all other concerns are equal) remains a risk minimization strategy.

Within crocodile farms, intentional hybridization has occurred to produce better quality skins, or faster growing offspring, but inclusive fitness is unknown. Knowing the species status of individuals within these mixed populations would allow for such comparisons if breeders could be paired in breeding pens. For the reintroduction effort, it was important to confirm that the captive population consists of mostly purebred C. *siamensis,* with some hybrid F1 and F2 individuals rather than an admixture that had gone on undetected (Allendorf *et al*., 2001). In contrast, a captive population in Thailand may had greater admixture between *C. siamensis* and *C. porosus* as it includes F2 hybrids and hybrid back crosses to both parental species (Chavananikul *et al*., 1994).

These results have been useful in identifying captive hybrids of the Siamese crocodile, *C. siamensis*. Using microsatellites to determine species status follows from previous genetic work using karyotyping to determine hybrid *C. siamensis* x *C. porosus* in crocodile farms of Thailand (Chavananikul *et al*., 1994). These two species differ in chromosome number and have substantially different karyotypes, and purebreds, F1 hybrids and F2 hybrids could readily be distinguished. Although*, C. siamensis* and *C porosus* have the different arrangement of chromosome types and a more detailed analysis would be required to recognize hybrids.

Of equal concern in reintroductions and augmentations is the possibility of breaking up locally adapted gene complexes through outbreeding among different source populations (Storfer, 1999). If this is true for *C. siamensis*, then it will remain an inherent problem for this and other crocodilian reintroductions, because crocodile farms typically have individuals originating from widely scattered populations. Records may not have been kept, individuals might not be marked for identification and breeders are typically put into large communal pens. One could argue that to avoid the problem of outbreeding depression, only the most productive lineages should be selected for reintroduction (if indeed this information is known). However, this runs the risk of selecting individuals who are best suited to captive rather than wild situations.  The extreme contrast between outbreeding depression versus hybrid vigour is something that undoubtably occurs make comparisons.

# CONCLUSION

Microsatellite DNA marker was developed from a wild caught *Crocodylus siamensis* are given step by step, from the screening of libraries enriched in $(AC)_{15}$ microsatellite repeats to the final characters of 18 microsatellite loci. Also published are primer sequences, estimates of allele size range, and expected heterozygosity in *C. siamensis* and in the closely related species *C. porosus* and *C. c. crocodilus* in which an optimal utility of the microsatellite markers was observed. Sequences of 22 positive clones were submitted in Genbank (accession numbers EF413033-EF413054). Eighteen microsatellite designed primers provided reliable amplifications and were evaluated in captive breeding crocodile farm for identify freshwater and saltwater crocodile. These microsatellite loci were showed specific band, multiple alleles and absent allele in 40 crocodiles.

Fourteen microsatellite loci (77% of total microsatellite loci) showed specific allele were considered as specific markers for identify species of crocodiles. These markers composed of one marker (CS-25) for *C. siamenis*, three markers (*CS*-4, *CS*-26 and *CS*-30) for *C. porosus*, nine markers *CS*-2, *CS*-7, *CS*-10, *CS*-12, *CS*-14, *CS*-17, *CS*-22, *CS*-24 and *CS*-35) for *C. c. crocodilus* and one marker (*CS*-20) that can identify all species.

Twelve microsatellite loci (66% of total microsatellite loci) showed polymorphic alleles in the same species that were considered as intra-specific markers. These markers composed of CS-4, *CS*-5, *CS*-10, *CS*-12, *CS*-17, *CS*-18, *CS*-21, *CS*-24, *CS*-26, *CS*-30, *CS*-32, and *CS*-35 might be useful in parternity testing and hybridization breeding

Seven microsatellite loci (38% of total microsatellite loci) showed as absent of allele in *C. c. crocodilus* that were considered as genus-specific markers. These markers composed of CS-4 (Figure 11), *CS*-5, *CS*-18, *CS*-21, *CS*-26, *CS*-30, and *CS*-32 might be useful in family testing.

It is therefore recommended that more microsatellite markers be used in these situations. The use of these microsatellite markers will broaden the scope of a breeding program, allowing progeny to be tested from adults maintained in large breeding lagoons for selection as future breeding animals.

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# APPENDIX

**Appendix Table 1** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413033).

```
1      CCAAGAGGCT  GCAGCACGGG AACAAAGAAG GGACAGAGAG GAGCTGAGAG   50
51     GTGGCAGAAA  AAGGTGGTGC GACCTGAGAG AGAGACAGAG ACTGGAATTT   100
101    AAGATGGGGA  AGGGACTGGG GCTCAGTAAA ATACAACCCA ACTCAGGGTT   150
151    ACAAATGACA  GTGGCTTTAT TGAACAGGGG AGATGGGGAC AATATCTTAT   200
201    TGCTTCCTTT  GCACACGCAC ACGCACACAT ACACACATGC ACACGTGCAC   250
251    ATGCGCACAC  ACATGCGCGC ACACACACAT GCGCGCGCGC ACACACACAC   300
301    ACACACACAC  GCACACACAC ACACTGGCAG CAGGGGTTAA AGAGGCTTGG   350
351    TGCAGGGGCT  GAAGTCCAGG AGGAGAGAGT CCAAATTGGA GCGGGAAATG   400
401    TGCAGGTGAT                                                410
```

**Appendix Table 2** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413034).

```
1      CCACAGAAAT AAAACTGGAA AGGGGAAGAA GTGGCAAACA TATAGAAAAA    50
51     AATTATACTC AGCGCATTAA GAGGTGCCCT CCACCCCATG AAATGGACAG    100
101    CAACAGAAAA GACAGGAAAA GACTGAGTAC TTACATTAGC CTCCCAACCC    150
151    TATCCCACGT CFGTCATCAT GGTAAATATT TTACCATGTG CAGGAATACA    200
201    CAATGTTTTT TAGAAAAATA ATTGGTTCCT CTCTGACCTC TCTCTCTCTC    250
251    TCTCCCACAG CCACTCTGCA TTTCCATCAG CACGAACGCT TCCTATGTCA    300
301    CACACTACCG CAACCCGCAT AAAACAACAT TTTT                     334
```

**Appendix Table 3** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413035).

```
1      CCTCAGCGAC CTGGCTCTAG GCCGGTGCTC ACTGCTGGAG AGGAGCACGC    50
51     GTGACGTACT GTTCCACGTG CGTGCATCGG ATGTGCACAG CAGGCAGAGG    100
101    AGTCTGGTCT CTTAGGTATT GCATCCGTGG CTGACAATGG AGCCTCTGGT    150
151    GCCAACCAGA GCTGGGAAAT ACTGCTTGGA GCTGGTGACA ACAGCCATCT    200
201    CTCTCTTGCG TGGAGTCCCT GAGCCAGTGG CTCTCAGCCT GGGCTGTGTG    250
251    AGATCCATGT AAGGGTGCTG TGGCATCTCA GCCCCGTGCA GACGCCTACA    300
301    CATCCCCCAT GAGAGAAACC CACACACATA GACACACATA GGTTTTTGCA    350
351    AGTGATCAGG TATACGCATA CACACTCCTA TCAGGTGCAC CTTCATGTAG    400
401    GTTAGGTACA CACACCAGAT ACATGAGGAT CAGGTTCATA CACACACTCC    450
451    CTTCATGGGG ACCAGGAGCA TGCGTGTGTG GATGCATCCA CACACACTCC    500
501    CCCCCTTCAT GTGGATTAGG AACAGGCGAA GGGGCAGATC ACGTACACAC    550
551    ACACACCCTT CACGTGGGTC AGGTACACAC ACACACACCC CTTCACATGG    600
601    CTCAAATACA CACACCTCTT CATGTGGAAC AGGTACTGAC ACACAAGCTG    650
651    ATCAGGTACA CACACAAACA CCACCTCACA TGGCTCAGGC ACACGTGCAT    700
701    GTGCACACGC ACACACACAC AGAGCAAACT TCCCTGCAAA TCCCTGTCCC    750
751    TGCTACCCAC TTCAAGCTGC TAATCTGTTT GCAACCTGAA GCTAAGCCTG    800
801    GCAGGGTCCC CCAGG                                          815
```

**Appendix Table 4** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413036).

| | | |
|---|---|---|
| 1 | ATCACCTGCA TATTTCTCCT TCCAATTTGA ACTCTCTCTC TCTCTTCTCC | 50 |
| 51 | CGGACTTCAG TCCCTGCACC AATCCTCTTT AACCCCTGCT ACCATTGTCT | 100 |
| 101 | GTGTGTATGT CTGTGTGTGC AAAGGAACCA ATAAAAATAT CATGTCACCA | 150 |
| 151 | TCTCCCCTGT TCAAATAAAA CCACTGTCAT TTGTAACCCT GAGTTGGGTT | 200 |
| 201 | GTGTTTTACT GAGCCCCGGT CCCTTCCTTA TCTTAATTTC TGCTTTCTGT | 250 |
| 251 | CTCTCTCTCT CGGCTCTCAG CTCAGTTCCC ATCACCTTTT GCCPCCTCTC | 300 |
| 301 | TGTCCCTTTT CTGTAGGCTC TCAGACCTGT TGCTTTCTGC AGACTCTCAG | 350 |
| 351 | CCTGGTTCCC ACTTTTTGCC TCTTGCCACC TCTTCCCAAT CCCTCTCTCT | 400 |
| 401 | CGCTGCCTAC ACTTTCTCGG GCTTGCCTGT GTTCTCCAGC CTCCCACCAT | 450 |
| 451 | CACAGCCCCA CTCCCAGTTT TCCTTTCCCC ACATACCTCT CGGCTCCCCT | 500 |
| 501 | GTAGCCTCCT TGCTCCAGCT CCAGCCTCCG AGCTTCTCCC TGGCATTGCT | 550 |
| 551 | CCTTTGCTCT GCCGG | 565 |

**Appendix Table 5** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413037).

| | | |
|---|---|---|
| 1 | ATCCCTCCTA CACACACACG CGCATGTGCA TATGCGCGCG CGCACACACA | 50 |
| 51 | CACACACACA CACACACAGA ACTAAATGTC TACCAACTGA ATTACCATAC | 100 |
| 101 | CTAGTTTGCC AGCGTTTATA GCCAACAAGA CACAAAGGAA TGACAAGGGT | 150 |
| 151 | AGCACCATGT CCACCTTTGA CTGTCCAGCC CTAATAACTA GGTACCTATT | 200 |
| 201 | TACACCAGAG TTCTCTTAAA GCATGTGATA TAAGTCCAAA ATAAGACTTA | 250 |
| 251 | CACTCATGCT TTTGAAGCCA TAGTAAGACA TCCTGTCTGC TCTACCACCA | 300 |
| 301 | TGCCTCTTCC TTGTGTGCAC CTATGTACAT ATATGAAGAC TAGCTGATTG | 350 |
| 351 | CCCATCAAGA ATGACCGGGG GAAGGGCTGG GATAGACTAG TGCCACCTAA | 400 |
| 401 | CGCCCCATGC TGCCGCTGTT CTGCCTCTTG CAAGGAGCAG GGTGGGGTCC | 450 |
| 451 | TTGGTGCCAC TTGCAGGGAG TGGGGTGGAG GAGCCAAGG | 489 |

**Appendix Table 6** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413038).

| | | |
|---|---|---|
| 1 | CCTCTAGTTG CTGAGTTAGT TACCACCTTC AAGAGCAGAA CCATAACACA | 50 |
| 51 | TGCACAGTGC ACACATACAC ACTCTTGGAT GATGTCCCCA GCACACCACC | 100 |
| 101 | ACAACCATCT GTAGTGGTTA AAAAAAAAAA AAAAAAAAGA CAGGCTGGCT | 150 |
| 151 | TTATTCATAT AGGCATAGCA GTACCATGCT GGTGCTCTAC AATCCAAAGG | 200 |
| 201 | ACAACAGGAA TTATTGTACT GTAGCTAGAC TCTCCATCAA GAAGACAAAA | 250 |
| 251 | GACACTATGG AGGGGACTAT GGAATGCATG CAGTGGCGAC CATGGGAAAA | 300 |
| 301 | GCCAATGCCT CCTGTAAGTC CTTTAGCCCA ATCTGTAGAA ACCTAGATTA | 350 |
| 351 | GAATGGGAAA AGTGAAATTT CGTCCCATTG CTTTTTCTAC GCTGCCACAT | 400 |
| 401 | ATGCCCCTCT AAGGAAAAAG CTTAGATGAA CCAATGTTCT CAAAAAGAGA | 450 |
| 451 | ATAATATTAG TTACTGCCTT GTAGCTGAGC CACCGCCCTG CACAAACAAA | 500 |
| 501 | AGGTGAAAAT AACGTAAAAC AGGTTTACAA CCATGCCAAT TCTTGGTGGC | 550 |
| 551 | ATTCTGAGCT GTTAATTAAA CAGGTGGCTA TTGTTTCAAA TAATATGGAA | 600 |
| 601 | CAGGTTAGAC ACCTTTCCAA ATGGCAAGAT CCACAGCTGT TCTTGTGCCC | 650 |
| 651 | CAGACTATAC AAGGCATGTT CAAACTTTAG GGCAGGTTT | 689 |

**Appendix Table 7** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413039)

| | | |
|---|---|---|
| 1 | ATCAATATTG TGCTGTTGGA CAGGAGGGTG TGACACAATT GCACATATAC | 50 |
| 51 | AAACACACAA ATCAGTTAGG TGCATACACA CACACACACT ACCAGTTCAG | 100 |
| 101 | GCACATACAC ATCCAAACCA GCCTAAGCAC ATGCAAACCT ATCACCAGTT | 150 |
| 151 | CAAGCACATA CACTATACAC TCCAAAACTT AGACACAGAT CACTAATTCA | 200 |
| 201 | TATATCATGC ACATAATGCC ACACACATGT GCGTGCGCGC ACACACACAC | 250 |
| 251 | ACACAACCCA CCTACACATA CACATGACTA AGTTCCTAAC TTGGATGGTA | 300 |
| 301 | CAGTGTGTGT GTGTGCTTGG GGTACCTGGG ATGCTTGGAG GAAGGTTCAG | 350 |
| 351 | GTGACAGAGA GAGAGTTAAA ATGTAGGGAG TGAAAGTTGC CAGAGCCGGG | 400 |
| 401 | ATTAGAATTG GTGGACTACA GAGAAGCTGG CTGAGGAACT GAGGCTTGGG | 450 |
| 451 | TTTACTTAAG GTAGACTCTG GCTGGAAACT GAGGTATTGG GGTGAAGATA | 500 |
| 501 | AGTGGTGGCA AGGAGGAGAC AGCCTGGAAA GAAGCTCAGG GGAAAAGGAA | 550 |
| 551 | AAGGCAGTGG GGATAAGATA GTGGCAAAGA AACAGGGGGG TTGGAGCCAG | 600 |
| 601 | AGAGGAGCTC AGGACAGGTG TTGGAGGTGG CAGTGAGCCA GAAGCAAGAG | 650 |
| 651 | AGGGAGAAAT GAGACAGAAG TTAAAGGGGA AACGAGCAGA GATTGGAGCA | 700 |
| 701 | GGG | 703 |

**Appendix Table 8** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413040).

| | | |
|---|---|---|
| 1 | CCTTCCGCCC AAAATAATTG CCCACCCCTG CCCCACACAC ACATTCACAG | 50 |
| 51 | CCTTCCACAC TTTTAGAAAT AACCCCCCCC CCACACACAC ACTTCAAAAA | 100 |
| 101 | TAGCATCTCA TGATATACTT TCCAAATCTC TTGATTTCTA AAAGTTTATC | 150 |
| 151 | TCATGACTTT TGACATGCTG GGCTTGGCAA TACTGAGCTC ATGATGCTGC | 200 |
| 201 | TTAGAGCACA GGGGCTGCTG CCAAGGTTCG GGGCTGCTCC TTGTTAGTGC | 250 |
| 251 | AGGGGGAGCC CGGGATTGAT | 270 |

**Appendix Table 9** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413041).

| | | |
|---|---|---|
| 1 | GGAGGGGCTC ACAGAACTTT TATATCAATT TGGTAGCCAA TCAACGTACT | 50 |
| 51 | CTCCGCAGAG AAATTTCTAC TCCGATTACC TCATGTCAGG TTCTGACTGA | 100 |
| 101 | GCATGCTCTC AGTCAGGAGC TGACACAATC TCAGCATCCC TCTCTGGGTA | 150 |
| 151 | AAGTAGGTCA GCAATCATTC ACAGCTCTCC AGGTCACCCA GGGCGCAAGG | 200 |
| 201 | ACTGGGTGAG CCTTGGAAAT TTCCACTCAC AGCTTCAAAC CGCCCATGTC | 250 |
| 251 | AGTTTAACCT CTTCTGTGCT CGCAGCAGAT ACTCAAAAAT TTCCATGGCA | 300 |
| 301 | CTAAACATAT TTTTTTCATG AAGTAATAAT GCGCGCGTGT GTCTGTGTAA | 350 |
| 351 | TGTGCGCACA CACACACATA TGTATATCTG CATATATATG TATGTTTGTG | 400 |
| 401 | TATGTATATA TATGGATATA TGTATACCTA TGAAATATAC AATACTGCCA | 450 |
| 451 | TATGTGAAGC TAAACAAGAC CTACAAGCAT CCTTTATAAT TCACACTTTA | 500 |
| 501 | TTTCATTTTT TAGTGCTGTA GTAAGCCAAC AGCTTATTTC TTTGTTCTTT | 550 |
| 551 | CCTCTATGCA GCTCAATTTA TATGTAGATA ATATTGCTTA ATTAGTTTAC | 600 |
| 601 | AGATTAGTGA TCCAGAT | 617 |

**Appendix Table 10** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413042).

1 AAAGTCTCAG TCACCTTCAC ATCTACAGAC TAAATAAAGG ACTGTAGGCT 50
51 GTGACAAAAT CCTGTAATGA CTGCATTTGT TAATATTGTT TTGCATCCAT 100
101 AGGTTTTATT TCCAAAACGG GAATAACCAA TAAAGCCAGG GAGAGAAAAA 150
151 GCCACACACA CACACACCAC CCATACACCA CACACCCCTC CCCCCAGATC 200
201 TGCAGTCACA TTCTGGTTGG TTATGTTTCT TACCACTCTA GTTGTAAAAA 250
251 GAATGTTGAA AAATGGCTGA CCCAGCCCCT CCTGTTGTCC TCTAGTTGAG 300
301 AAGAAGTAGT TCTTGCAAAT CCAATACTGG GGCTTGAGTA GAATAAAAGG 350
351 AGTATGACCA TAGCATCTTT ACACTCTGTT GATCTCTGCT GGAACATCCC 400
401 TTTCAGAGTG GTAGACAGAC AATTATACTA CAAGCCATAA GACAGATCTG 450
451 AATTATGTCA AAGGTAGG 500

**Appendix Table 11** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413043).

1 CCCACACCCC AGAGCCGGGC AGGTCGGGGC AGGCTGCGCC CTGGCACCCC 50
51 ACACATTGGG AACCCAGGAC GCAGGAGAGC TCACAGGAGA CAGCAGTGTG 100
101 TGTGTGTGTG TGTGTGTGTG TGTGTAAAGT CTGGGTAAAT TAGCGTGTAT 150
151 CTCAGATTCT TACGTGTCGT GTGTGTGTGT GTGTGTGTCT GTGTGTGTGT 200
201 GTGTGTGTGT GTGTGTGTGT GTGTGTGTCT GTGTGTCCAC GACATATGGA 250
251 TTCCCTGTGT GTGAGTGTGT GGGACACCTG GGTGCTCTGT GTGTGTGTGT 300
301 GTGTGTGTGC GCGCGCACAT GTCCCGCACA CCTGGGTTCT GTGTATGTGT 350
351 GTCCATGTCC CAAAGTCCTC TGTTGTGTGT GCGTGTGTGT ATTCCAGATC 400
401 TGCA 404

**Appendix Table 12** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413045).

1 CCATGCAGTC TGGTACACTT GTTCCTGCTG CTAGGAAAGT AATGCCCATG 50
51 ATAACATCAG GAATCCCCAG AGTGTATCCA ATCACAGTAA CCTGTGCAGT 100
101 AAGGAAAAAC AGAGCCATGT GTACTAACTG GGAAGTCTCT CTTAGACTAA 150
151 TCCTGAAATT CCAATACAGT TTTTCACTTT CATTTCCAGA GGCTGGCATG 200
201 ACAAAGATAA CACACACACA CACACACACA CACACACACA CACGCGCATA 250
251 ATGAACCACA ACTGTAGCAA TAACTTGACT AAAAATGGTT TGCACACACC 300
301 TGTGTAATGA AACAAAACTG TAGCAACAAC CTGACTAAAA ATGGCTGGTA 350
351 TGAGG TGTAT AAAATTTTAG ACAGACAAGG GTCTTTTGGT GAATCTGAT 400

**Appendix Table 13** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413047).

1 CCAATGCTCA GGATCTCACT ATTGCGTGGT TAGACAGGAG GGCATGGTGT 50
51 ACAACTGCAC ATGCACACAT AAAAAATCAA TTAAACACAC ACTAGCTGGG 100
101 TGCATACACC GCTATTAGCC ATACACACTC ACACTACCAT CTCCAGCACA 150
151 TACAATATAC ACCTCAAAAG TTAGATACAA ATCACTAATT CATGTATCAT 200
201 GCACACAATG CCATATACAT ATACACACAC ACACACACAC ACACACACAC 250
251 ACGCACACTC TCTCACACTA GCAACTCAGA TACATGAACG TTCAAAATAA 300
301 TTAGTCTAGG TTCACGCACA TGACACACAC CAAATTTAGA ATCAGTTACC 350
351 AACACCTGAA CATTCAATAC ACATATGTGG ATTACTAATT CATATACCAC 400
401 ACACACACAC AATGAT 404

**Appendix Table 14** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413048).

| | | |
|---|---|---|
| 1 | ATCACCTGCA CATTTTCCCC TCCAATTTGG ACTCTCTCTC TCTCTCTCTT | 50 |
| 51 | TCTCTCCTCT AGGACTTCAG CCCCTGTACC AAGCCCCTTT AACACCTGCT | 100 |
| 101 | GCCAGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGA ATGAACCAAT | 150 |
| 151 | AAGATATTGT GTCACCATCT CCAGCCCTTC TCCAGCTCCT GGCACTGGCA | 200 |
| 201 | GCCCCTCCCC TGGTTCCAAG AGCCAGCCCC CAGTTTCGTC TGCAACCTCT | 250 |
| 251 | CTACCTCCCC TGCACCCTCT CCTAGTTTCC TTCTCCCCCC TTACCTTTTG | 300 |
| 301 | GCTCCCCTGT AGCTCTAGCT TCGGTCCCAG CCTCTGTGCT GGCTTCTCTC | 350 |
| 351 | CGGCATCACT CCCCTGTTCT GAGGGCTTTG GGG | 400 |

**Appendix Table 15** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413049).

| | | |
|---|---|---|
| 1 | CCATTAAGGA TCAACTTTCA GCCTGGGATA GGTAGATACT AGCTTCATAC | 50 |
| 51 | ATCCTCCTAC AATTTGGACT CTCTTTCTCT CCCTCTCTCT CCTCCTGGAC | 100 |
| 101 | TTCAACTGCT GCACCTAGCC TCTCTAACCC CTGCTGCCAC TGTGTGTGTG | 150 |
| 151 | TGTGTGTGTG TGTGTATGTG TGTGGGTGTG GGTGCAAGGG AAGCAATAAG | 200 |
| 201 | ACATTGTGTC ACCATCTCCC CTATTCAATA AAATCACTGT CATTTGCAAC | 250 |
| 251 | CCCAAGTTGG GTCATATTTT TCTGAATCCC AGTCCCTTCC CTCTGCTAGT | 300 |
| 301 | TTCCCAATCT CCTCCCAATT CTGTCTCCCC TTTTCTGCCA CCTCTCAGCC | 350 |
| 351 | TCTCCTTGCT TTTCCTGCTG CATTTCAGCC TCCCATGCCA TCTACCGCCT | 400 |
| 401 | TTTCCCTGTG AATTTCTGCT GCCTTTCCCT GCTTTTCCAC AGCCCTGCTG | 450 |
| 451 | CCTTTCAGTC TCTCCTGCCA CTTTTCCTGT GATTTTCTGC TGTCTGTTTT | 500 |
| 501 | CAGGCTTCCC CTGCACCTTC TGTAGTCACC CCACCCCCAG CTTCCCAGCT | 550 |
| 551 | CATCATGGCT GTCCTGCTGT TTTCTCCAGC AGCTGGAGCC TTTCTCCAGC | 600 |
| 601 | TCCCAGCACC CACAGCCACT CCCCGCAGTT TCTCTATATG CGTCAGAGAC | 650 |
| 651 | ATCCTCTGGC TCCCCATACA CAGAGTCCCT CTTTAACACC CACACTTTCC | 700 |
| 701 | CTTAGTCCCA TTTTCCCTCT CCCCACTGAC CTTTTCAGCT TCCCCCGTAG | 750 |
| 751 | CTCTGGCTCC ATCCAGGCCC AGACTCTGTG CCGGCATCTC TCCGATGTTC | 800 |
| 801 | TGCAGGCTTT GGGCATTGTC TTTTAATCAA CTGAT | 850 |

**Appendix Table 16** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413050).

| | | |
|---|---|---|
| 1 | GAGAGTACAC AAACACCAGC AGACTTTCCC TATGTCTGAA AAGGGTGTTT | 50 |
| 51 | GTGCCAAAAG CTCGAAAAGA ACAATTTTGT CAACTGTTAC ATTGGTCTGT | 100 |
| 101 | TAAAAGATAT CACATCTACC CAAAGAACCT TGTCTACCAA CACTCCCCCC | 150 |
| 151 | TCACACGCGC GCCCGCACAC ACACACACAC ACACACACAC ACACACACAC | 200 |
| 201 | TCTTGGATGT TACAGTATCT TGGCTTCTAA CAGTTTAGGT CACCTATAAG | 250 |
| 251 | AGACAGCAAC GGAATTCAGT CACGCAACGG AATTCAGT | 300 |

**Appendix Table 17** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413051)

| | | |
|---|---|---|
| 1 | CCTGGGATCC CCCATGCACC CCATGCCCTA CCACACAACT GTGAAAGCAG | 50 |
| 51 | GAAGTCAGAA AACCATACAT ACACACACAC ACACACACAC ACACACATAC | 100 |
| 101 | ACAAGTAGCA GAAGCAATTC AAAAGTGAAA GCAATTTGAA GGCGTGAACT | 150 |
| 151 | ACAAAAAAGT CACACCACCA TCTGGTGGGC AAAGAGGCTC ATTACACATC | 200 |
| 201 | TTTGAATGCC TTTGTGTTTG CATGCAACAA ATCAATGGAC ACAACACTTT | 250 |
| 251 | AATTCCCAGC AAGCCTAAGT AAACTATGTC TGAGGAGTTT TAGTTTAATG | 300 |
| 301 | CACAAGAAAG ACTTTT | 316 |

**Appendix Table 18** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413052)

| | | |
|---|---|---|
| 1 | CGACTCTCCC AGCACTGCCC CCACTGTCCC TACTATCTCT CTTTCCCCAC | 50 |
| 51 | CACCCTTGTC CTTTGGGTTC CTATTTTTCT CTCCTTCTTT CTGCTTGTGC | 100 |
| 101 | TCCTTCATGC TTTCTCCATG GCCTTTTACA GCATCTGATG AAGTGAGCTT | 150 |
| 151 | ATTTCACACA CACACACACA CACACACACA CACACACAGA TACAGACAGA | 200 |
| 201 | CATCCAGGGA AGTTAGATCA CATTAAAAAC AACCACCTAA CTGAACTTAG | 250 |
| 251 | TTCACACAGG TACAGACCTT GCACTTAGAT TGG | 283 |

**Appendix Table 19** Nucleotide sequences from recombinants DNA of clone (accession numbers EF413054).

| | | |
|---|---|---|
| 1 | AATCAGTTAC CAGTTACCAA CACCTGTATA TTCAATACAC ATATGTGGAT | 50 |
| 51 | TACTAACTCA TATACCACAC ACATACCCAA TTATATCTAT ATATATATAT | 100 |
| 101 | ATATATATGT GTGTGTGTGT CTCTCTCTGA ATTCACTAAT TTACACACAT | 150 |
| 151 | ACCACCTACC TATACATACA TGCAAGTAAC TTCCTAAGTT GGGTATTGTG | 200 |
| 201 | ATATGTATGT ATGTGTTTGC TGTACTTGGG ATACCTGGGG AAAGGTTAAG | 250 |
| 251 | GTAAGAAAGT ACAACTGTAG GGGGTTAAAG TTACC | 285 |

**Appendix Table 20** Alleles frequency of microsatellite loci developed from wild *C. siamensis* testing in *C. siamensis*, *C. porosus*, *C. c. crocodilus*, and Hybrid *(C.s+C.p)*, Alleles frequency (Freq.), observed and expected heterozygosity (Ho and He).

| Locus | Alleles size | *C. siamensis* | | | *C. porosus* | | | *C. c. crocodilus* | | | Hybrid (*C.s*+*C.p*) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He |
| *CS*- 4 | 196 | 0 | 0.61 | 0.38 | 1 | 0 | 0 | 0 | 0 | 0 | 0.75 | 0.50 | 0.38 |
| | 200 | 0.50 | | | 0 | | | 0 | | | 0 | | |
| | 210 | 0.50 | | | 0 | | | 0 | | | 0.25 | | |
| *CS*- 5 | 186 | 0 | 0.55 | 0.57 | 0 | 0.50 | 0.61 | 0 | 0 | 0 | 0 | 0.50 | 0.625 |
| | 188 | 0 | | | 0.63 | | | 0 | | | 0.25 | | |
| | 190 | 0.03 | | | 0 | | | 0 | | | 0 | | |
| | 200 | 0.60 | | | 0 | | | 0 | | | 0.50 | | |
| | 202 | 0.23 | | | 0 | | | 0 | | | 0 | | |
| | 204 | 0.05 | | | 0 | | | 0 | | | 0 | | |
| | 210 | 0.09 | | | 0 | | | 0 | | | 0 | | |
| | 215 | 0 | | | 0.37 | | | 0 | | | 0.25 | | |
| *CS*- 10 | 198 | 0 | 0.24 | 0.50 | 0 | 0.25 | 0.53 | 0.50 | 0 | 0.5 | 0 | 0.50 | 0.38 |
| | 210 | 0 | | | 0 | | | 0.50 | | | | | |
| | 220 | 0 | | | 0.37 | | | 0 | | | 0 | | |
| | 224 | 0 | | | 0.63 | | | 0 | | | 0.75 | | |
| | 240 | 0.52 | | | 0 | | | 0 | | | 0.25 | | |
| | 250 | 0.48 | | | 0 | | | 0 | | | 0 | | |
| *CS*- 12 | 198 | 0 | 0.21 | 0.12 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| | 200 | 0.87 | | | 1 | | | 0 | | | 0 | | |
| | 204 | 0.13 | | | 0 | | | 0 | | | 0 | | |
| *CS*- 17 | 151 | 0 | 0.35 | 0.34 | 0 | 0 | 0 | 0.40 | 0.80 | 0.52 | 0 | 0 | 0 |
| | 172 | 0.24 | | | 0 | | | 0 | | | 0 | | |
| | 192 | 0.76 | | | 1 | | | 0 | | | 0 | | |
| | 281 | 0 | | | 0 | | | 0.60 | | | 1 | | |

**Appendix Table 20** (Continued)

| Locus | Alleles size | *C. siamensis* | | | *C. porosus* | | | *C. c. crocodilus* | | | Hybrid (*C.s*+*C.p*) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He |
| *CS*- 18 | 240 | 0.75 | 0.28 | 0.24 | 1 | 0 | 0 | 0 | | | 1 | 0 | 0 |
| | 247 | 0.25 | | | 0 | | | 0 | | | 0 | | |
| *CS*- 21 | 192 | 0.21 | 0.38 | 0.19 | 0 | 0 | 0 | 0 | | | 0.25 | 0.50 | 0.38 |
| | 200 | 0.79 | | | 1 | | | 0 | | | 0.75 | | |
| *CS*- 24 | 270 | 0.58 | 0.14 | 0.50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 278 | 0.42 | | | 1 | | | 0 | | | 1 | | |
| | 395 | 0 | | | 0 | | | 1 | | | 0 | | |
| *CS*- 26 | 240 | 0.59 | 0.55 | 0.49 | 0 | 0 | 0 | 0 | | | 0 | 0.50 | 0.38 |
| | 252 | 0.41 | | | 0 | | | 0 | | | 0.25 | | |
| | 255 | 0 | | | 1 | | | 0 | | | 0.75 | | |
| *CS*- 30 | 206 | 0 | 0.24 | 0.44 | 0.88 | 0.25 | 0.23 | 0 | 0 | 0 | 0.25 | 0.50 | 0.63 |
| | 210 | 0.60 | | | 0.12 | | | 0 | | | 0.5 | | |
| | 212 | 0.40 | | | 0 | | | 0 | | | 0.25 | | |
| *CS*- 32 | 208 | 0.29 | 0.45 | 0.68 | 0 | 0.25 | 0.53 | 0 | 0 | 0 | 0 | 0.50 | 0.38 |
| | 216 | 0.61 | | | 0 | | | 0 | | | 0 | | |
| | 222 | 0.10 | | | 0.63 | | | 0 | | | 0.25 | | |
| | 230 | 0 | | | 0.37 | | | 0 | | | 0.75 | | |
| *CS*- 35 | 170 | 0 | 0.38 | 0.47 | 0 | 0.25 | 0.53 | 0.50 | 0.25 | 0.50 | 0 | 0 | 0 |
| | 175 | 0 | | | 0 | | | 0.50 | | | 0 | | |
| | 204 | 0.36 | | | 0 | | | 0 | | | 0 | | |
| | 210 | 0.64 | | | 0.63 | | | 0 | | | 1 | | |
| | 214 | 0 | | | 0.12 | | | 0 | | | 0 | | |
| | 230 | 0 | | | 0.25 | | | 0 | | | 0 | | |

**Appendix Table 20** (Continued)

| Locus | Alleles size | *C. siamensis* | | | *C. porosus* | | | *C. c. crocodilus* | | | Hybrid (*C.s*+*C.p*) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He | Freq. | Ho | He |
| *CS*-2 | 230 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | | | 1 | 0 | 0 |
| | 232 | 0 | | | 1 | | | 0 | | | 0 | | |
| | 235 | 0 | | | 0 | | | 0 | | | 0 | | |
| | 249 | 0 | | | 0 | | | 0 | | | 0 | | |
| | 275 | 0 | | | 0 | | | 0 | | | 0 | | |
| *CS*- 14 | 270 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | | | 1 | 0 | 0 |
| | 276 | 0 | | | 1 | | | 0 | | | 0 | | |
| *CS*- 15 | 200 | 0 | 0 | 0 | 0 | 0 | 0.50 | 0.25 | 0 | 0.50 | 0 | 0 | 0 |
| | 210 | 0 | | | 0.50 | | | 0.25 | | | 0 | | |
| | 214 | 0 | | | 0 | | | 0 | | | 0 | | |
| | 220 | 1 | | | 0.50 | | | 0.25 | | | 1 | | |
| | 230 | 0 | | | 0 | | | 0.25 | | | 0 | | |
| *CS*- 20 | 184 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.50 | 0 | 0.50 |
| | 188 | 0 | | | 1 | | | 0 | | | 0.50 | | |
| | 190 | 0 | | | 0 | | | 1 | | | 0 | | |
| *CS*- 22 | 174 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 180 | 1 | | | 1 | | | 0 | | | 1 | | |
| *CS*- 25 | 170 | 0 | 0 | 0 | 0 | 0 | 0.50 | 1 | 0 | 0 | 0 | 0.50 | 0.63 |
| | 180 | 1 | | | 0 | | | 0 | | | 0.25 | | |
| | 200 | 0 | | | 0.25 | | | 0 | | | | | |
| | 206 | 0 | | | 0.63 | | | 0 | | | 0.25 | | |
| | 210 | 0 | | | 0.12 | | | 0 | | | 0.50 | | |

# CIRRICULUM VITAE

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