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Georg-August-Universität Göttingen

Genetic Conservation and Utilization of Indigenous Livestock in Northern Thailand

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DEDICATION

'TO MY BELOVED FATHER, MOTHER AND SISTERS,
TO RESPECTED PROFESSORS AND TEACHERS,
AND TO ALL FARMERS IN THAILAND'

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8. Charoensook R., Gatphayak K., Brenig B. & Knorr C. (2007) Analysis of mtDNA D-loop sequence polymorphisms and phylogenetic relationships in Thai indigenous pigs (oral presentation). Proceedings of The German Society for Animal Production e.V. (DGfZ 2007). University of Hohenheim, Stuttgart, Germany.

LIST OF ABREVIATIONS

A	: Adenosine
AFLP	: Amplified Fragment Length Polymorphism
AI	: Artificial Insemination
AMOVA	: Analysis of Multi-variance
AM-PCV	: Blood Pack Cell Volume in the Morning
AM-RR	: Respiratory Rate in the Morning
AM-RT	: Rectal Temperature in the Morning
APCV	: Average Blood Pack Cell Volume
ARR	: Average Respiratory Rate
ART	: Average Rectal Temperature
AnGR	: Animal Genetic Resources
bp	: Base Pairs
C	: Cytisine
^{o}C	: Degree Celsius
CD	: Chiang Dao (Amphoe)
CR	: Chiang Rai (Province)
CS	: Chiang San (Amphoe)
CSB	: Conserved sequence Blocks
ddH2O	: Deionized and Demineralized Water
DLD	: Department of Livestock Development, Thailand
D-loop	: Displacement Loop Region (Control Region)
DNA	: Deoxyribonucleic Acids
dNTP	: Deoxynucleotide Triphosphate
EDTA	: Ethylene-Diamine-Tetra-Acetic Acid
Fa	: Fang (Amphoe)
FAO	: Food and Agriculture Organization
FCR	: Feed Conversion Ratio
FT	: Fak Ta (Amphoe)

G	: Guanine
GDP	: Gross Domestic Product
GLM	: General Linear Model
hr	: Hour
HSP	: Heat Shock Protein
HSP90AB1	: Heat Shock Protein 90 AB1 gene
HTC	: Heat Tolerant Coefficient
JT	: Jhom Thong (Amphoe)
kb	: Kilobase
kg	: Kilogram
LP	: Lamphun (Province)
MAS	: Marker Assistant Selection
MFA	: Ministry of Foreign Affair, Thailand
mg	: Milligram
MH	: Mae Hongson (Province)
min	: Minute
ml	: Milliliter
mM	: Milimolar
MOAC	: Ministry of Agriculture and Cooperatives, Thailand
mRNA	: Massenger RNA
MT	: Mountain cattle
mtDNA	: Mitochondrial DNA
MW	: Molecular Weight
NCM	: Northern part of Chiang Mai
NP	: Nam Pad (Amphoe)
NSO	: National Statistical Office, Thailand
OAE	: Office of Agricultural Economics
O.D.	: Optical Density
OK	: Om Koi (Amphoe)
PCR	: Polymerase Chain Reaction
PCV	: Pack Cell Volume
PM-PCV	: Blood Pack Cell Volume in the Afternoon

PM-RR : Respiratory Rate in the Afternoon
PM-RT : Rectal Temperature in the Afternoon
QTL : Quantitative Trait Loci
RAPD : Random Amplified Polymorphic DNA
RFLP : Restriction Fragment Length Polymorphism
RNA : Ribonucleic Acid
rpm : Rotation per Minute
RR : Respiratory Rate
RT : Rectal Temperature
SAS : Statistical Analysis System Software
SCM : Southern part of Chiang Mai
SD : Standard deviation
SE : Standard Error
SPSS : Statistical Package for the Social Sciences
SNPs : Single Nucleotide Polymorphisms
SSCP : Single Strand Conformation Polymorphism
T : Thymidine
THG : Thai Haplogroup
TNH : Thai Native Pig Haplotypes
TNP : Thai Native Pigs
TWH : Thai Wild Boar Haplotypes
TWB : Thai Wild Boars
UPGMA : Unweighted Pair-Group Methods Arithmetic Average
VC : Viang Chai (Amphur)
VNTR : Variable Number Tandem Repeats
W : Watt
WL : White Lamphun cattle
w/v : Weight by Volume
μg : Micrograms
μl : Microliter

SUMMARY

In recent years livestock production in Thailand has switched from backyard systems to industrialized husbandry. In parallel, exotic livestock was imported to improve production performance and for economically important traits. Indigenous livestock has therefore gradually been used for crossbreeding and was finally replaced completely by exotic commercial breeds. However, these breeding strategies oppose the concepts of sustainability and resource management and will lead to the threatening risk of losing genetic identity and diversity of indigenous breeds.

For that reason, the overall goals of this study are to gain genetic information at the molecular level that is indispensable to conserving Thai pigs and cattle breeds as well as to define their potential as genetic resources. In particular, this study is aimed at:

(1) to investigate the mtDNA composition and to determine the genetic diversity of pigs indigenous to Northern Thailand,

(2) to assess the phylogeny of Thai indigenous pigs, to compare them with further Asian and European pigs and to clarify their origin of domestication,

(3) to compare the genetic background of Thai indigenous pigs with commercial pigs used for meat production in Thailand and with selected Chinese pig breeds (i.e. Jiangquhai, Luchuan, Minzhu, Rongchang, Yujiang and Tibetan),

(4) to search for sequence polymorphisms within the bovine *HSP90AB1,* to record physiological responses against heat stress and to describe putative associations between them in three cattle breeds used in Thailand.

The complete mtDNA control region (1264-1324 bp depending on the individual) was comparatively sequenced to determine the degree of shared haplotypes, the population structure and the phylogenetic relationships within Thai pig populations. For that, samples of 72 Thai native pigs and 11 Thai wild boars were collected in six regions (i.e. Mae Hongson, Southern and Northern part of Chiang Mai, Chiang Rai, Nan and Uttaradit provinces) of Northern Thailand. In total 36 nucleotide variations leading to the formation of 24 different haplotypes were described (TNH01 to TNH02 and TWH01 to TWH04). The phylogenetic tree was separated into two main clades: a European (E) clade and an Asian (A) clade with further Asian subclades (AS1, AS2 and THG).

Twenty-three of the 24 mtDNA haplotypes were integrated into the Asian clade of the phylogenetic tree and eight of them recapitulated another major cluster of haplotypes (THG). One haplotype (TNH01) fit to the European clade of the phylogenetic tree.

Average pairwise distances of 0.0136 ± 0.0029 (between AS2 and THG), of 0.0109 ± 0.0023 (between AS2 and AS1) and of 0.0084 ± 0.0023 (between THG and AS1) resulted in estimates for the time since divergence of 90,000 - 496,000 years between mtDNA clade AS2 and clade THG, 72,000 - 397,000 years between clade AS2 and clade AS1, and 56,000 - 306,000 years between clade THG and clade AS1. The data implies that THG and AS1 diverged from the AS2 clade, but also that AS1 is evolutionarily older than THG. In addition, our present study suggested that Thai native pigs are closely related with Thai wild boars, but are also distinctly separated from them enough and can be traced back to the common Asian ancestor.

An additional analysis using 510 bp of the sequenced mtDNA incorporated the THG haplotypes to clade MTSEA (mountainous and Southeast Asian distribution) to form haplogroup MTSEA-THG. Recently, MTSEA was renamed in MC3. MC3 contains only signatures of pigs scattered across the Indo-Burma Biodiversity Hotspot (IBBH), a region including Thailand to the Kra Isthmus. The assignment of the 15 porcine Thai haplotypes to cluster AS1, supports the hypothesis of a shared common ancestors with the Chinese domestic pigs, but the formation of the separate MTSEA-THG clade is also most putatively an indication for a further independent domestication event in Southeast Asia (SEA) in the past. All haplotypes of haplogroup MTSEA-THG have revealed unique and previously unknown nucleotide signatures at positions 24 (nucleotide A) and at positions 183 (nucleotide C) that differentiate them from all other porcine mtDNA haplotypes.

The genetic background and genetic diversity at the nuclear DNA level of the Thai indigenous breeds was analyzed using 26 microsatellite markers. Thai indigenous pig populations have a high genetic diversity being mirrored in relatively high scores for the effective heterozygosity (He; 0.71) and the effective number of alleles (Ne; 3.71). Furthermore, the genetic distances, the pairwise proportion of different alleles, the neighbour-joining tree and the multidimensional analysis indicated a close genetic relationship between the Thai indigenous and the selected Chinese pigs. Contrary to that Thai pigs are distinctly different from European pigs. Nevertheless, a genetic

introgression traced back to European commercial breeds is evident in some of the Thai native pigs. The genetic analyses clearly point out that Thai native pig populations are unique genetic resources.

Thailand is a tropical country and lies in the hot and humid climatic zones of the world. The environmental heat, resp. the heat stress, is most detrimental to cattle production and welfare which can be visible, for example, a hindrance of feed consumption, a decreased milk production and a limited reproduction performance. Heat shock proteins act as molecular chaperones that have preferentially been transcribed in response to severe perturbations of the cellular homeostasis, such as heat stress. Thus, the traits respiration rate (RR), rectal temperature (RT), pack cell volume (PCV), and the individual heat tolerance coefficient (HTC) were recorded as physiological responses on heat stress (environmental temperatures) in *Bos taurus* (crossbred Holstein Friesian; HF) and *Bos indicus* (Thainative cattle: White Lamphun; WL and Mountain cattle; MT) animals. The 47 apparently healthy not lactating females were randomly selected and kept at the experimental farm of the Chiang Mai University in Thailand. RR and RT were measured in the morning (8:00 am) and in the afternoon (2:00 pm), two weeks per month for four consecutive months (September to December) to achieve 8 observations per animal. During the experimental time an averaged surrounding temperature of 22 °C with 94% relative humidity was measured in the morning. The records for the afternoon were 34°C and 68% relative humidity.

Polymorphisms of the heat shock protein 90-kDa beta gene (*HSP90AB1*) were evaluated by comparative sequencing of animals representing *Bos taurus* and *Bos indicus*. Nine SNPs were identified, i.e. three in exons 10 and 11, five in introns 8, 9, 10, 11, and one was located in the 3'UTR. The exon 11 SNP g.5082 C>T led to a mis-sense mutation (alanine to valine), the further SNPS proved to be silent. The calculated genetic heterozygosity based on allele frequencies suggests a higher genetic diversity of Thai native cattle (MT = 0.326 and WL = 0.307) compared to the *Bos taurus* animals (HF = 0.071). During the period of extreme heat (in the afternoon) RR and RT were in each of the three breeds elevated, whereas the PCV decreased. MT and WL were superior in all physiological traits compared to HF. The association analysis using a stepwise regression revealed that the T allele at SNP g.4338T>C within intron 9 improved the heat tolerance ($p < 0.05$) of the animals. Allele T was exclusively found in

WL animals and to 84% in MT. HF cattle revealed an allele frequency of only 18%. The study indicates breed specific physiological responses to heat stress. Here, polymorphisms within *HSP90AB1* were not causative for the physiological responses, however, the results propose that this gene is an attractive candidate for heat tolerance, and should at least be used as a genetic marker to select appropriate breeds for hot climates.

CHAPTER 1

General Introduction

General Introduction

1 Current situation of livestock production in Thailand

A major structural change in livestock production has occurred in the past 20-25 years in Thailand. Although private sector innovations such as improved breeds, feed technology, housing, farm management, and contractual arrangement have been the prime sustainers of growth, export opportunities and rapid domestic and regional economic growth during the period from 1985-1995 were the essential catalyst (FAO 2002). The livestock industry has grown in close proximity to Bangkok, and the heavy concentrations of animals are causing environmental stress. Farm sizes have become significantly larger over the past ten years. This expansion is made possible by imported technology and increased domestic demand as a result of rapid economic growth. Swine and cattle development have been driven by domestic market demand, and have been significantly affected by policy factors-regulation of slaughterhouses and subsidies (FAO 2002). In this section, the general information about Thailand, Thai agricultural economics and livestock husbandry system focusing on swine and cattle production will be discussed.

1.1 General facts about Thailand

Thailand lies at 6^{o} N and 20^{o} latitude in Southeast Asia and covers an area of 513,120 square kilometers. It is bordered to the north by Myanmar and Laos, to the east by Laos and Cambodia, to the south by the Gulf of Thailand and Malaysia, and to the west by the Andaman Sea and the southern extremity of Myanmar. The country is geologically divided into four ecological region parts: the Northern part (mountainous region), the Northeastern part (Khorat Plateau area, bordered to the east by the Mekong river), the Central part (predominately the flat Chao Phraya river valley) and the Southern part (the narrow Kra Istmus). Eighty percent of the country lies below an altitude of 500 m with only 5% above 1,000 m. The general weather conditions throughout the country are those of a monsoonal tropical climate and remain hot throughout the year. The average

temperature is about 29 ºC, reaching 35 ºC in April to 17 ºC in December in Bangkok (capital city) from. There are three seasons in Thailand: the winter season (November to February), the summer season (March to May) and the rainy season (June to October) (Na-Chiangmai 2002; MFA 2010).

According to the National Statistical Office (NSO), the population of Thailand is currently 67,070,000 inhabitants. The Gross Domestic Product (GDP) was US$ 3,939 per capita in 2009 (NSO 2010a). Thailand is an agricultural country, around 34% of the households throughout the country working in agriculture and 93% of them living in rural areas. A major activity in the agriculture area is the cultivation of crops (54%) and integrated crop-livestock farming (35%). Fifty-three percent of the cultivated area has been used for rice cultivation (NSO 2010b). The major livestock in Thailand are pigs, chicken and cattle.

1.2 Economic values of agriculture and livestock production

Thailand is a major export nation of agricultural products to countries all over the world. Agriculture's share of GDP in 2009 was around 9.2%. Within the agricultural sector plants provided approximately 68% while the livestock sector is only a relatively small part of the overall agricultural sector and contributed only for 17% in year. The agricultural sector in Thailand has been undergoing a substantial transformation to non-traditional crops away from rice and cassava. It has been shifting towards high valued products. Para rubber, frozen chicken and shrimp products have become important, particularly for export markets. According to the Office of Agricultural Economics (OAE), the major export products in 2009 are rice (US$ 4,784 million), Para rubber (US$ 3,595 million), shrimp products (US$ 2,588 million), frozen chicken (US$ 1,304 million) and cassava products (US$ 1,296 million) (OAE 2010).

In 2010 the Office of Agricultural Economics (OAE 2010) has estimated that Thailand's share of agricultural in the economy will decrease by 0.9%. The two major contributing causes are a serious drought and the infestation of crop pests since the early months of the year 2010 which was accentuated with heavy floods which came later. Consequently, the impact upon most of the major crops is a decline in production, as yearly crop production index falls by 2.1% from the year before. However, the overall

prices of the crops are favorably high especially for Para rubber, cassava and palm oil. For rice alone, even though farmer prices received are lower than in 2009, they have still been favorably maintained at high levels, contributing to a 22.8% increase of the farmers' received price index. Livestock sector, due to favorable price incentives coupled with no serious livestock epidemic outbreak followed by a bright export trend, livestock production is expected to be on the rise by 1.5%. Livestock production such as the dairy and the beef production are almost insignificant components of the Thai economy in terms of aggregate output (FAO 2002). Furthermore, growth of the fishery sector is expected to be 1.2% due to its production expansion in the first half of 2010 as a result of growing demand for raw material supplies used in processing for export purposes. Therefore the fishery prices and the entries sector will continue to grow (OAE 2010).

1.3 Pigs and beef cattle husbandry in Thailand

At present, livestock production in Thailand is growing very quickly and plays an important role in food production. It has been shifting from backyard animals and integrated crop-livestock farming systems to industrial livestock farming enterprises. But this development differs between livestock species. Rapid growth has occurred in pig and poultry production. Broilers, layers and pigs are mainly produced for export market and raised by large agribusiness companies (FAO 2002; Na-Chiangmai 2002; OAE 2010). The challenges for pig production in Thailand are increasing to close the big gap between demand and production and are reached by intensification of production system towards high-input, high-output systems. Contrary to the pig production, the importance of beef cattle and buffaloes is still low, in spite of the fact that they are mostly raised by smallholders in rural areas and not by companies.

1.3.1 Pig production

The development of pig production started in the 1960's when the first group of exotic pig breeds were imported by the Department of Livestock Development (DLD) from the United Kingdom. These were Large Whites, Tamworth and Berkshire breeds. Later,

Landrace and Duroc Jersey pigs were imported from the United States (DLD 2010). Before these exotic breeds were introduced, farmers relied on the relatively slow growing native pigs that had the desirable quality of not needing much in the way of traded inputs (FAO 2002). Since 1981 pork breeding began to be industrialized in Thailand. Thus, indigenous native pigs have been increasingly mated with imported breeds to improve their performance for economically important traits. Native pigs have gradually become crossbreds and have been finally replaced by European commercial breeds as the meat delivering end product in the pork industry (Rattanaronchart 1994).

Nowadays, like in other major swine-producing areas of the world, there has been a change from small farms to large farming enterprises. This trend will continue and is expected to lead to improved quality pork and to raise the interest of overseas' importers. Ten large operators control most of the increase in current production and the outlook for development is significant. Groups of agribusiness companies such as Charoen Pokphand (CP), Betagro, Laem thong and Mittraparp are integrated and account for more than 20% of the swine production in Thailand. These operations are fully automated and have increased efficiency of production, which that will make them competitive on the world import market.

Concerning production amounts of pigs, the total commercial breeding swine population in 2009 was 2,542,069 animals (Table 1.1). The sow population is estimated at 906,099 animals. These sows wean an average of 17 pigs/sow/year (DLD 2010). The primary swine-producing area is the central region with approximately 57% (4,669,535 heads) of the country's pig population (8,537,703 heads). The Southern part has the least number of pigs, possibly reflecting the higher costs of pig fattening because of a shortage of feed in this region. An alternate explanation could be that the Southern part of Thailand has a relatively high Muslim population for whom consuming pork is prohibited. Most of the pork produced in Thailand is consumed domestically because of the presence of foot and mouth disease (FMD) in some of the producing areas in Thailand. Export markets are limited to Hong Kong, Vietnam and Singapore. Finished (processed) pig meat based products are more widely exported (FAO 2002; DLD 2010).

Table 1.1 Numbers of pigs separated by number of animals (head) and farmers (households) in 2009.

Region	Native breeds		Breeding and barrows		Total	
	Animals	Farmers	Animals	Farmers	Animals	Farmers
Northern	218,406	50,365	1,145,564	47,943	1,363,970	98,308
Northeastern	142,116	26,033	1,340,001	63,022	1,482,117	89,055
Central	36,910	4,671	4,632,625	19,500	4,669,535	24,171
Southern	57,459	7,933	964,622	28,322	1,022,081	36,255
Total	454,891	89,002	8,082,812	158,787	8,537,703	247,789

Source: Modified from DLD (2010)

1.3.2 Native pigs

Contrary to commercial pigs, Thai native pigs are predominantly raised by communities in the Northern part with almost half of the country's native pig population (Table 1.1). The average number of pigs per household is 4.3 heads. Especially smallholders in the hill tribe communities traditionally raise a few indigenous pigs due to local customs and religion. Animals are sacrificed at special celebrations such as New Year and weddings (Rattanaronchart 1994; Nakai 2008a, b). However, small pig populations without any scrutinized breeding programmes are always at risk of losing genetic diversity and identity (Charoensook *et al.* 2009a; Charoensook *et al.* 2009b).

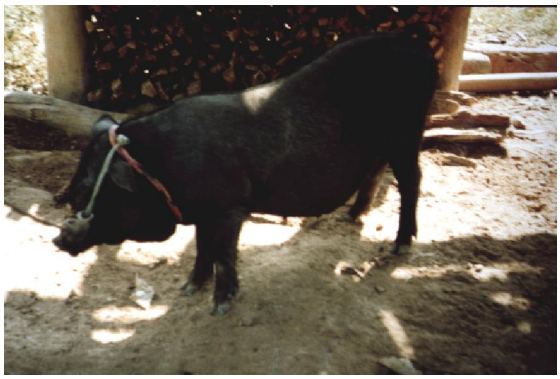
Thai native pigs are classified as lard type pigs. They grow slowly and their reproduction rate is low. They, however, adapt well to hot and humid climates, tolerate low quality feed, and are probably resistant to, for example, the foot and mouth disease as well as internal parasites (Rattanaronchart 1994). The characterization of Thai native pigs has been described by the domestic animal diversity information system (DAD-IS 2010) of the Food and Agriculture Organization (FAO). Native Thai pigs are classified into four "breeds" according to their physical appearances and the regions where they are predominant i.e. Raad (or Ka Done), Puang, Hailum and Kwai (Table 1.2 and Figure 1.1).

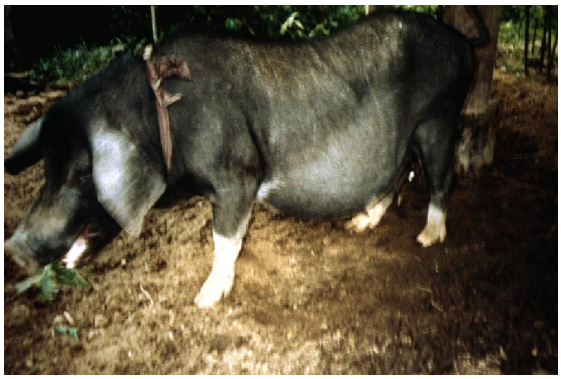
Table 1.2 Phenotypic classification of four Thai native pigs

Name	Weight [a] (kg)	Specific phenotypes	Number of teats	Litter sizes	Predominant in Thailand
Raad	60-70	Black hair coat color, shot body, small head, small and erect ears, long and straight snout.	9-12	5-6	Lower Northeastern
Puang	120-130	Black and wrinkled skin, large thick ears. Similar to Chinese Taihu pigs.	---	6-7	Upper Northeastern
Hailum	110-120	Black and white hair coat color, black color at the head, back, and rump, white on the belly and legs, short and straight snout, small and erect ears. Similar to Chinese Hainan pigs.	10-14	7-8	Central, Eastern and Southern
Kwai	130-150	Black hair coat color, white legs, long and straight snouts, larger ears, white ring around a black cornea.	10-12	6-7	Northern

Modified from Rattanaronchart (1994) and DAD-IS (2010)
[a] Average mature weight of female and male pigs.

In Northern Thailand, some pigs were kept and bred by hill tribes. Hence, some authors have classified them as an independent group (Rattanaronchart 1994). They have a narrower head, a longer snout and a shorter body compared to Thai native pigs from the lowlands. Hill tribe pigs can be classified into two types: the small black type (similar to Raad or Ka Done pigs) and the black and white type (similar to Hailum and Kwai pigs). However, 70% of the hill tribe pigs belong to the small black type. Large-eared pigs found in the Thunghuachang district of Lamphun province, which are probably crossbreds of hill tribe pigs and Chinese Meishan pigs, are more prolific than hill tribe pigs. However, nowadays, it is difficult to determine real characteristics specific for each pig breed (Rattanaronchart 1994; Charoensook *et al.* 2009a; Charoensook *et al.* 2009b).


Raad (or Ka Done)


Puang


Hailum


Kwai

Figure 1.1 Four breeds of Thai native pigs (Rattanaronchart 1994; DAD-IS 2010).

1.3.3 Beef cattle production

According to the Department of Livestock Development (DLD 2010), the numbers of beef cattle increased from 4,635,741 to 8,595,428 between 2000 and 2009 (Table 1.3). The increase was due to the policy of the Thai government to encourage farmers to raise beef cattle in an effort to reduce the number of imported beef (GPRD 2010). Several activities aiming to increase beef cattle production initiated by the Thai government proceeded such as the Royal initiated Cattle-and-Buffalo Bank project in 1978, the Beef Cattle Farm Promotion in the Northeastern Region in 1989 and the One Million Beef Cattle Households Promotion in 2004 (DLD 2010).

Table 1.3 Number of beef cattle (head) from 1999 to 2009 in each region of Thailand

Year	Region				
	Northern	Northeastern	Central	Southern	Total
1999	875,403	2,219,437	855,232	685,669	4,635,741
2000	943,251	2,522,961	849,237	585,165	4,900,614
2001	1,025,750	2,573,233	1,022,264	606,357	5,227,604
2002	1,132,292	2,910,823	936,075	570,995	5,550,185
2003	1,297,460	3,078,149	984,069	556,645	5,916,323
2004	1,326,987	3,693,782	1,001,425	646,138	6,668,332
2005	1,636,851	4,092,206	1,296,820	770,395	7,796,272
2006	1,564,797	4,316,945	1,315,270	839,041	8,036,057
2007	1,953,406	4,501,769	1,516,298	876,919	8,848,392
2008	1,847,601	4,931,389	1,553,668	779,435	9,112,093
2009	1,677,932	4,655,444	1,496,033	766,019	8,595,428

Source: Modified from DLD (2010)

In 2009, the average number of cattle per household for the whole country was just 6.2 heads. This indicates that the majority of beef cattle are owned by smallholders. The main region is the Northeastern region where 54 % of Thailand's beef cattle were found in 2009 (Table 1.4).

Table 1.4 Numbers of beef cattle separated by number of animals (head) and farmers (households) in 2009.

Region	Native breeds		Exotic/Crossbred		Total	
	Animals	Farmers	Animals	Farmers	Animals	Farmers
Northern	1,008,686	108,091	669,246	59,098	1,677,932	165,223
Northeastern	3,083,410	623,931	1,572,034	331,991	4,655,444	898,305
Central	710,758	58,534	785,275	58,097	1,496,033	114,228
Southern	639,561	163,357	126,458	38,936	766,019	191,962
Total	5,442,415	953,913	3,153,013	488,122	8,595,428	1,369,718

Source: Modified from DLD (2010)

The number of purebred and crossbred cattle was 3,153,013 heads compared to 5,442,415 heads of native cattle, which indicates the genetic potential of them. Beef cattle in Thailand are produced by extensive grazing systems rather than confined feedlots or control grazing. Village farmers, who generally raise a small number of ruminants, usually use small areas besides crop fields for grazing in addition to paddy fields after the harvest (Kawashima 2002; DLD 2010).

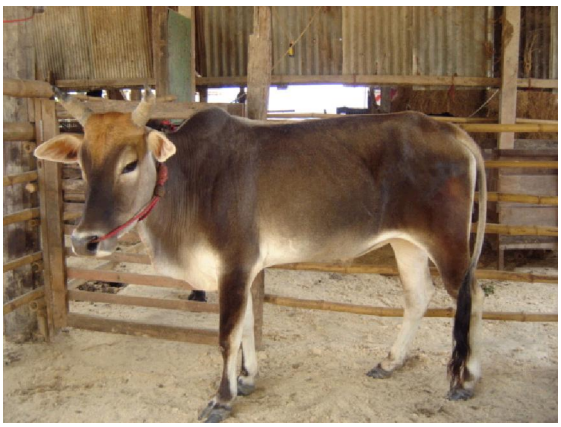
1.3.4 Native cattle

Thai native cattle are classified as *Bos indicus* cattle and were predominantly used as draught animals in the past. They have accompanied Thai people for a long period of time and are now adapted well to local environments (Intaratham 2002). The Northeastern part of the country is also the most important area in terms of native cattle production with an average of five heads per household. Thai native cattle are mainly kept under extensive grazing. During the dry season the animals graze in the forests or are fed only rice straw. Thai native bulls weigh between 300 and 450 kg and cows 200 - 300 kg on average (DLD 2010). Although Thai native cattle are small framed and display a low growth rate, they seem to have a good adaptability to low quality feed. They are also heat tolerant and resistant to parasites. The low energy requirements and the efficient utilization of low quality roughage without protein favors their survival under a severe feeding environment (Intaratham 2002; Kawashima 2002; DLD 2010).

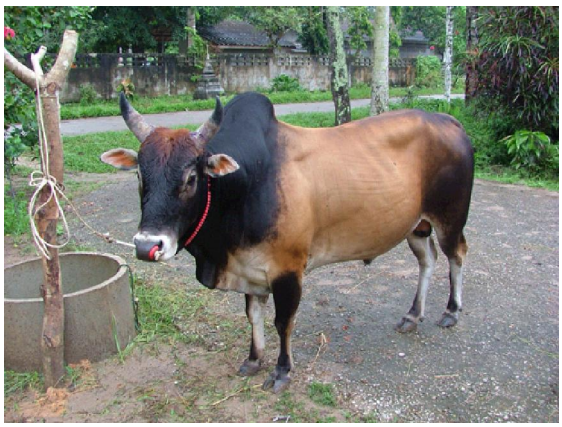
Thai native cattle are categorized into four ecotypes i.e. the Northern ecotype (White Lamphun), the Northeastern ecotype, the Central ecotype and the Southern ecotype (Figure 1.2). This classification is confirmed by the study using phenotypic information of cattle kept on government research farms according to their original region by using a cluster analysis with a 75% coefficient of determination. However, there is no genetic information with respect to the difference between the ecotypes (Intaratham 2002; Akkahart 2003).

Northern ecotype

Northeastern ecotype



Central ecotype



Southern ecotype

Figure 1.2 Four ecotypes of Thai native cattle.

In Northern Thailand, the White Lamphun and the Mountain cattle are two widely spread native cattle breeds. They show a rather high rate of fertility, are tolerant to a poor quality of natural grasses, and are also well adapted to internal and external parasites. They are also resistant to diseases such as Anaplasmosis. They adapted well to hot and humid climate (Rattanaronchart 1998). White Lamphun show an entirely white phenotype as a pink skinned cattle. They are classified as an endangered-maintained breed (with probably fewer than 1,000 breeding females). Their origin is still unknown, but it has been a popular breed among Northern Thai populations. The name is derived from the Lamphun province where the breed was prevalent (Rattanaronchart 1998; DLD 2010). Mountain cattle varies in color (red brown, white gray or black) and are probably the smallest (150-200 kg mature

wt.) breed among the Thai native cattle breeds. They were mainly raised in the mountainous areas (Rattanaronchart 1998).

The above mentioned performance advantages of native Thai cattle have been overshadowed by the large body size of imported exotic breeds. Therefore, indigenous cattle have been neglected and crossed with zebu cattle (*Bos indicus*) such as Brahman as well as with several *Bos taurus* breeds (Chantalakana & Skunmun 2002; DLD 2010). These were mostly imported into the cattle population by mean of frozen semen as Charolais, Hereford, Simmental and Shorthorn for crossbreeding with the native cattle (FAO 2002; Intaratham 2002).

1.3.5 Other major livestock in Thailand

Other major livestock in Thailand are dairy cattle, buffaloes and chickens. Dairy production in Thailand has high production costs due to high feed prices; low milk production efficiency due to poor management and poor quality animals; the use of low fat powdered milk to produce drinking milk rather than raw milk because of lower costs; and the poor quality of raw milk. Additionally, the high price of land has led to a shortage of forage while employment opportunities away from the farm for the younger generation have led to labor shortages. According to DLD, Thailand had 1.36 million buffaloes in 2008, a decrease of 1.8 million from 1999. The greatest proportionate, increase, however, occurred in the Northeastern region, while the slowest growth occurred in the Southern region (DLD 2010). The increased mechanization which has occurred within Thai agriculture has resulted in the replacement of buffalo on many farms by tractors and other mechanical implements; this trend is likely to continue. The Thai chicken production system has had a great success. Broiler production is completely integrated with feed milling companies and production is mainly for export markets. The layer industry began in 1950 at Kasetsart University, but rapid development only began in the mid-1970s when commercial layer hybrids were introduced from Western countries. Modern management is used in the layer industry, and each bird produces 250 to 260 eggs per year (DLD 2010).

2 Genetic diversity and evaluation of livestock

Genetic diversity is generated by mutations, and the frequency of different allele changes due to migration, selection and by chance. Genetic diversity of livestock represents the heritable variation within and between populations. Populations may be either the entire species or a specific collection of individuals within a species such as a breed, a strain, a line, or even a herd/flock (Rege & Okeyo 2006). Genetic diversity is required for populations to evolve and to cope with environmental changes. A loss of genetic diversity is often associated with inbreeding and a reduction of reproductive fitness. In addition, genetic diversity and the evaluation of domestic animals have attracted increased attention worldwide. Consequently, the International Union for Conservation of Nature (IUCN) recognizes the need to conserve genetic diversity as one of three global conservation priorities (Frankham *et al.* 2002). Thus, a better understanding of the mechanisms which cause the genetic diversity is a priority needed to manage livestock populations. Worldwide efforts are undertaken to conserve livestock diversity. Monitoring the number of breeds, their population sizes and degree of endangerments is coordinated by the FAO on a global level. The FAO State of the World's Animal Genetic Resources report shows that roughly one-third of all breeds are considered to be at the risk of extinction (FAO 2007b).

2.1 Assessment of genetic diversity and phylogeny

DNA sequence variants may result in amino acid substitutions within the protein encoding the locus. Such protein variations may result in functional biochemical or morphological dissimilarities that cause differences in the reproductive rate, the survival or the behavior of individuals. Moreover, these genetic variations are spread through the population by recombination events due to sexual reproduction (Frankham *et al.* 2002). Therefore, genetic diversity has been measured for many different traits, including continuously varying (quantitative) characters, for deleterious alleles, for proteins, for nuclear DNA loci, for mitochondrial DNA (mtDNA) and for chromosomes. Genetic diversity is typically described using parameters to reflect the amount of polymorphism, the average heterozygosity, the allelic diversity and the genetic distances (Table 1.5).

Table 1.5 Terminology used to describe genetic diversity

Terminology	Description
Genome	The complete genetic material of a species, or individual (all of the DNA, all of the chromosomes)
Locus	A segment of DNA, or an individual gene
Alleles	Different forms of the same locus that differ in the DNA sequence, e.g. alleles A, a, B, b, etc.
Genotypes	The combination of parental alleles present at a locus in an individual, e.g. AA, Aa or aa
Haplotypes	Parental alleles at several loci on the same chromosome, e.g. Abc
Homozygous	An individual with two copies of the same allele at a locus, e.g. AA or aa
Heterozygous	An individual with two different alleles at a locus, e.g. Aa
Allele frequency	The frequency of an allele in a population
Monomorphic	Lacking genetic diversity; a locus in a population is monomorphic, if it has only one allele present in the population.
Polymorphic	Having genetic diversity; a locus in a population is polymorphic, if it has more than one allele present in the population
Proportion of polymorphism (*P*)	Number of polymorphic loci / total number of loci sampled
Average heterozygosity (*H*)	Sum of proportions of heterozygotes at all loci / total number of loci sampled. Typically, expected heterozygosity (H_e) are less sensitive than observed heterozygosity (H_o). In random mating populations, H_e and H_o are similar
Allelic diversity (*A*)	Average number of alleles per locus
Co-dominance	Situation where all genotypes can be distinguished from the phenotype, i.e. AA, Aa, aa can be distinguished
Genetic distance	A measure of the genetic difference between allele frequencies in populations is based on many loci and can be used for reconstruct phylogenetic trees, e.g. Nei's genetic distance (Nei 1972).

Source: Modified from Frankham *et al.* (2002)

Furthermore, data on the genetic diversity has been used to reconstruct phylogenetics on the order of genome rearrangements, so-called breakpoint phylogenies (Blanchette *et al.* 1997). Phylogeny is the study of genetic relationships among various groups of organisms (e.g. species, population) that descend from a common ancestor. This approach can be used to compare any two existing organisms, no matter how greatly they may differ in their morphological traits (Salemi & Vandamme 2004).

The methods used to construct phylogenetic trees from molecular data can be classified into two types depending on the type of data used. Firstly, classification occurs according to whether the method uses discrete character states or a distance matrix of pairwise dissimilarities. Secondly, classification depends on according to whether the method clusters operational taxonomic units (OTUs) stepwise, resulting in only one best tree, or considers all theoretically possible trees. Table 1.6 lists the state of the phylogenetic tree construction and tree analysis methods, classified according to the above mention strategies used. Computer programs such as PHYLIP (Felsenstein 1995), MEGA (Tamura *et al.* 2007) or PAUP (Swofford 2002) can be used to construct the phylogenetic tree.

Table 1.6 Phylogenetic analysis methods and their strategies

	Exhaustive search	Stepwise clustering
Character State	Maximum parsimony (MP)	
	Maximum likelihood (ML)	
Distance Matrix	Fitch-Margoliash	UPGMA
		Neighbor-joining (NJ)

Source: Modified from Salemi & Vandamme (2004)

2.2 Molecular markers of genetic characterization in livestock

The application of molecular markers in the study of genetic diversity has evolved very rapidly since the mid-1960s. The dominating protein electrophoresis approaches within the field of population genetics and evolutionary biology were replaced by DNA analysis in the late 1970s primarily through the use of restriction enzymes. In the 1980s DNA fragment approaches and mitochondrial DNA sequence analyses

become more popular. More recently, the introduction of PCR-mediated DNA genotyping or sequencing has provided the first rapid and easy access to the ultimate genetic data (Rege & Okeyo 2006).

At present, several molecular markers have been widely used for genetic diversity and phylogenetic analyses in livestock. These include microsatellites analysis, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), single nucleotide polymorphisms (SNPs), direct sequencing, mitochondrial DNA (mtDNA) analysis and Y-chromosome specific markers (Toro *et al.* 2009; Groeneveld *et al.* 2010). In this part mtDNA, microsatellites and SNPs analysis focusing on pigs and cattle are discussed.

2.2.1 Mitochondrial DNA (mtDNA)

MtDNA is maternally inherited without recombination. Therefore, the number of nucleotide differences between mitochondrial genomes directly reflects the genetic distance that separates them. Secondly, it mutates 5-10 times more frequently than nuclear DNA, thus allowing the study of the divergence between wild and domestic populations under the short time scale of domestication (Toro *et al.* 2009).

In pigs, initial mtDNA studies showed that European and Chinese pigs were domesticated independently from European and Asian subspecies of wild boar (Giuffra *et al.* 2000; Larson *et al.* 2007a). But later studies suggested, however, at least seven domestication events across Eurasia and East Asia (Larson *et al.* 2005; Larson *et al.* 2007b; Wu *et al.* 2007). These studies also suggested the occurrence of introgression of Asian domestic pigs into some European breeds during the 18th and 19th centuries. Larson *et al.* (2007b) demonstrated that multiple domestication occurred at different centers on the island of Southeast Asia and Oceania. Domestic pigs of Near Eastern ancestry were introduced into Europe during the Neolithic period. The European wild boar was also domesticated at this time. Once domesticated, European pigs rapidly replaced the introduced domestic pigs of Near Eastern origin throughout Europe. Moreover, a recent study hypothesized, five new cryptic domestication events from three geographical location namely India (MC1), peninsular SEA (MC2, MC3, MC4), and the coast of Taiwan (MC5) (Larson *et al.* 2010).

In cattle, one of the first contributions of DNA research to reconstruct the domestication was a comparison of the mtDNA of taurine and indicine cattle (Bradley *et al.* 1996). The divergence of their control regions implied separate domestication events, which most likely started around 8,000 years BC in Southwestern Asia and the Indus valley, respectively (Zeder *et al.* 2006). Zebus were probably imported into Africa after the Arabian invasions in the 7th century (Bradley *et al.* 1998). Interestingly, the discovery that African zebus carry taurine mtDNA implies that African zebus were the result of crossing zebu bulls with taurine cows (Bradley *et al.* 1998). Moreover, mtDNA polymorphisms have revealed several other aspects of the early differentiation of taurine cattle. The predominance of one taurine mtDNA haplogroup (T1) in Africa (Troy *et al.* 2001) and a new haplogroup in Eastern Asia (T4) suggested two other regions of domestication (Mannen *et al.* 2004; Kantanen *et al.* 2009). However, complete mtDNA sequences showed that T1 and T4 are closely related to the major T3 haplogroup, so their predominance probably reflects founder effects in Africa and Eastern Asia respectively (Achilli *et al.* 2009). The T3 mtDNA haplogroup is predominant in most European and Northern Asian breeds (Kantanen *et al.* 2009) and is one of the four major haplogroups (T, T1, T2 and T3) in Southwestern Asia. By contrast, in the African taurine cattle haplogroup T1 is dominant, which is rare in Southwestern Asia. These observations are in line with a Southwest-Asian origin of European cattle, confirming the paleontological evidence of a gradual introduction of domestic cattle in Europe from Southwestern Asia (Zeder *et al.* 2006; Groeneveld *et al.* 2010).

2.2.2 Microsatellite markers

There are several types of nuclear DNA markers. Microsatellites have been the markers of choice to study genetic variation in recent years. Based upon sites in which the same short sequence is repeated multiple times, they present a high mutation rate and have a co-dominant nature. This makes them appropriate for the study of both within and between-breed genetic diversity. According to the FAO and the International Society of Animal Genetics (ISAG), microsatellite panels have been established for the genetic characterization of pigs and cattle (FAO 2004).

The porcine panel consists of 27 and the bovine of 30 polymorphic markers. In a collaborative EU project (PigBioDiv1), 58 European populations including local breeds, national varieties of international breeds, privately owned commercial populations and the Chinese Meishan breed as an out-group, were genotyped for 50 microsatellite markers. The microsatellite data showed that the individual breed contributions to between-breed diversity ranged from 0.04% to 3.94% of the total European between-breed diversity. The local breeds accounted for 56%, followed by commercial lines and international breeds (Ollivier *et al.* 2005). The ongoing project PigBioDiv2 covers 50 Chinese breeds and investigates mtDNA and Y-chromosomal regions in addition to the microsatellite data of the European breeds (Groeneveld *et al.* 2010). Trait gene loci and markers will also be analyzed to seek insight into the functional differences between breeds. The first results of the microsatellite-based analysis using pooled DNA samples indicate that Chinese breeds reveal a higher degree of genetic variability than the European breeds both within and between breeds (Megens *et al.* 2008; Groeneveld *et al.* 2010).

Bovine microsatellite data (Cymbron *et al.* 2005; Li *et al.* 2007; Medugorac *et al.* 2009) and AFLP fingerprinting results (Negrini *et al.* 2007) are in line with an endemic expansion of agriculture and cattle raising from Southeastern to Northwestern Europe (Groeneveld *et al.* 2010). Cymbron *et al.* (2005) observed that the correlations between genetic and geographical distances are different for Mediterranean and Northern cattle breeds; it is proposed that this reflects the separate Neolithic migrations along the Mediterranean coasts and the Danube, respectively. A larger set of microsatellite data (Lenstra 2006, 2008) indeed indicates a separate position of the Mediterranean cattle, but divides the Transalpine cattle into two different clusters of breeds: Central-European (Alpine, Southern-French) and Northern European. Genotypes from 30 microsatellites for 69 European breeds were used to test formal criteria of conservation (Lenstra 2006). The popular Weitzman method, based on genetic distances, favors highly inbred populations even if these have been derived recently from other populations. Ranking of conservation priorities on the basis of marker-estimated kinships was less influenced by inbreeding and favored Mediterranean breeds. These breeds have indeed a relatively high degree of molecular diversity, which next to phenotypic uniqueness is an obvious argument for conservation (Groeneveld *et al.* 2010).

2.2.3 Single Nucleotide Polymorphism (SNP)

SNPs are point mutations in the genome sequence, predominantly bi-allelic and highly abundant throughout the genome. They are widely used in the study of animal genetics and breeding because they have the potential to detect both neutral and functional genetic variations because (although most of them are located in non-coding regions) some correspond to mutations inducing changes in expressed genes (Amaral *et al.* 2008; Fang *et al.* 2009; Toro *et al.* 2009).

Fang *et al.* (2009) investigated genetic variations in the melanocortin receptor 1 (MC1R) gene among 15 wild and 68 domestic pigs from both Europe and Asia to address the genetic determination of why coat color is so much more variable in domestic animals than in their wild ancestors. They found that all mutations were silent in wild animals suggesting a purifying selection. However, nine of ten mutations found in the domestic pigs resulted in altered protein sequences, suggesting that early farmers intentionally selected for novel coat color. Amaral *et al.* (2008) evaluated linkage disequilibrium (LD) and haplotype block structures in 15 to 25 individuals from each of 10 European and 10 Chinese breeds genotyped for 1,536 SNPs in three genomic regions. The LD extends up to 2 cM in Europe and up to 0.05 cM in China. The authors suggest two possible explanations: either the European ancestral stock had a higher level of LD, or modern breeding programmes have increased the extent of LD in Europe. The haplotypic diversity using SNP has also been studied in another material investigating the polymorphism of porcine *IGF2* gene (Ojeda *et al.* 2008). The results show that selection can be observed and analyzed in the making by comparing different breeds that represent distinct stages of the selective process. Moreover, there is no evidence that, overall, domestication reduced genetic variability in the IGF2 region with respect to current wild ancestors of the pig (although a complete selective sweep is found in some very lean breeds such as Pietrain) (Groeneveld *et al.* 2010).

The SNP data (McKay *et al.* 2008; Gibbs *et al.* 2009) will reveal more about the history of European cattle. SNPs emphasize the zebu-taurine divergence and hence also the difference between Podolian and other European cattle (Negrini *et al.* 2007). Large-scale SNP analysis showed that in several breeds LD extends further than in humans, but is hardly detectable at distances over 200 kb (Gautier *et al.* 2007; Gibbs *et al.* 2009).

These data also suggested a rapid recent decrease of the effective population size of domestic cattle (Ginja *et al.* 2009; Kantanen *et al.* 2009).

Large numbers of SNPs, however, are required for precision, it is said as a rule of thumb that about six SNPs are equivalent to one microsatellite (Toro *et al.* 2009). In addition, another critical aspect is their discovery, usually by sequencing techniques. Nevertheless, it seems that they are becoming the markers of choice because of increasing automation coupled with low costs. Several large-scale projects are currently carried out to identify SNPs in livestock. In the near future, new technologies such as high throughput SNP typing or even whole-genome sequencing are likely to revolutionize our knowledge about the diversity and uniqueness of breeds, with the ultimate objective of gaining a complete understanding of the molecular basis of functional diversity (Groeneveld *et al.* 2010).

3 Livestock genetics and breeding for climate change

Genetic diversity is required for animals to evolve and to cope with environmental changes. Moreover, genetic diversity in livestock is critical for food security and rural development. It allows farmers to select stock or develop new breeds in response to changing conditions, including climate change, new or resurgent disease threats, new knowledge of human nutritional requirements, and changing market conditions or societal needs (Hoffmann 2010). All of the effects will be most acute in developing countries (where the increase in demand is expected to be greatest) and will occur at a rate faster than increases in production. Moreover, the effect will occur where climate change is projected to have its greatest impact (FAO 2003, 2006a). Climate change and global warming always affect the products and services provided by agricultural biodiversity. The report of the Intergovernmental Panel on Climate Change (IPCC) describes the predicted impact of climate change on ecosystems and agriculture. Several papers provide a general overview of the expected impact of climate change on livestock production by physiological stress (Zwald *et al.* 2003; Hoffmann 2010).

Heat stress is an important factor in determining specific production environments already today (Zwald *et al.* 2003). Temperature is predicted to increase globally, with

reduced precipitation in many regions, particularly in already arid regions. In livestock, heat stress is known to alter the physiology, reduce the reproduction and production, and increase mortality. Livestock water requirements increase with temperature. Heat stress suppresses appetite and feed intake; thus feeding rations for high-performing animals need to be reformulated to account for the need to increase nutrient density. Although the direct effects of climate change on the animals are likely to be small as long as temperature increases do not exceed 3 °C, projections suggest that further selection for breeds with effective thermoregulatory control will be needed. This calls for the inclusion of traits associated with thermal tolerance in breeding indices, and more consideration of genotype-by-environment interactions (G × E) to identify animals most adapted to specific conditions (Hoffmann 2010).

Most indigenous breeds are, however, not well characterized and their adaptation includes not only heat tolerance but also their ability to survive, grow and reproduce in the presence of poor seasonal nutrition as well as parasites and diseases. For example the use of heat-resistant individuals in sheep breeding program as a main strategy to improve animal welfare and productivity in hot climates. Various physiological and blood parameters differ between local and exotic cattle breeds in Brazil (McManus *et al.* 2009). Several Latin American cattle breeds with very short, sleek hair coats were observed to maintain lower rectal temperatures, and research in the major 'slick hair' gene, which is dominant in inheritance and located on bovine chromosome 20, is ongoing (Olson *et al.* 2003; Dikmen *et al.* 2008). Selection for heat tolerance in high-output breeds based on rectal temperature measurements and inclusion of a temperature–humidity index (THI) in genetic evaluation models are promising. Different parameters, such as THI or dry-bulb temperature measurements, are used as indicators for heat stress (Finocchiaro *et al.* 2005; Bohmanova *et al.* 2007; Dikmen & Hansen 2009). Different THI definitions were found to be preferable in the USA, depending on the extent of natural and artificial evaporative cooling (Freitas *et al.* 2006; Bohmanova *et al.* 2007). The genetic variance caused by heat stress was substantial at high THI (Ravagnolo & Misztal 2002; Hoffmann 2010). However, very few studies have been conducted on the genetic background of indigenous animals in hot climates. The genetic characterization of them as a model will be a benefit for studying the adaptive physiologic processes augmented by heat stress. Future research still needs

firstly, use of endocrine regulations as a means of improving thermal tolerance and secondly, identification of genes associated with heat tolerance and sensitivity. These diverse tasks require a coordinated collaboration of nutritionists, physiologists, biotechnologists and animal breeders.

4 Management strategies for animal genetic resources in Thailand

The Food and Agriculture Organization (FAO) defined genetic resources as those populations that show the highest genetic differences within a species and/or show unique alleles and allelic combinations. The term animal genetic resources (AnGR) is used to include all animal species, breeds and strains that are of economic, scientific and cultural interest to humankind in terms of food and agricultural production for the present or the future. Another equivalent term increasingly used is livestock genetic resources. There are more than 40 species of animals that have been domesticated (or semi-domesticated) during the past 10 to 12 thousand years which contribute directly (through animal products used for food and fiber) and indirectly (through functions and products such as draft power, manure, transport, store of wealth etc.). Common species include cattle, sheep, goats, pigs, chickens, horses, buffalo, but many other domesticated animals such as camels, donkeys, elephants, reindeer, rabbits etc. are important to different cultures and regions of the world (FAO 2006b; Rege & Okeyo 2006; FAO 2007b).

The conservation and utilization of indigenous AnGR has recently become concepts of higher importance. Conservation of animal genetics is now vital for sustainable management of these resources. This can be accomplished by the preservation of endangered and valuable breeds, selection programmes which will restore genetic diversity in industrial breeds, or the cryo-conservation of gametes, embryos and somatic cells of the existing gene pool (Ajmone-Marsan 2010). The utilization of indigenous AnGR will be a benefit to breeding programmes of high production livestock under tropical climates.

Thailand as well as other international countries, has agreed upon the Agenda 21 of the United Nations Conferences on Environment and Development in 1992, to conserve

the biological diversity and the global environment. The National Environment Board of Thailand has established the action plan for sustainable conservation of biological diversity in 1998. Strategies have been outlined to strengthen the capacity for sustainable use of the environment and natural resources as well as standard criteria for the conservation of biological resources that are applicable to the country (Khumnirdpetch 2002; DLD 2010). The Department of Livestock Development (DLD) under the Ministry of Agriculture and Cooperatives (MOAC) is responsible for livestock health and production. The activities regarding the conservation of AnGR are described in the national plans for biological diversity. The strategies are as follows: (i) to enhance capacity building, (ii) to increase the ability to conserve effectively, (iii) to create the public awareness in conservation of AnGR, (iv) to conserve the diversity of breed, population and genetic resources, (v) to minimize harmful activities to the biodiversity, (vi) to encourage the conservation and the use of the national resources including both the environment and the culture and (vii) to encourage the cooperation between the agents both nationally and internationally. All activities focus on the indigenous AnGR (Khumnirdpetch 2002; DLD 2010).

As stated above, the livestock production system in Thailand has changed from a backyard animal to industrial husbandry and also has species of importance. Most of the animals used for food production were imported exotic breeds or their crossbred with indigenous animal. Although the indigenous animals have a large genetic diversity and there were some efforts to characterize some species on the molecular level, genetic information to confirm their original identity is still lacking (Khumnirdptech *et al.* 2000; Khumnirdpetch 2002; Charoensook *et al.* 2009b). Breed characterization based on local names and phenotypic descriptions that have been used for a long time cannot clarify the admixture or gene introgression in populations. Therefore, a well-characterized population and appropriate breeding program must be determined to describe the uniqueness of the resources or a sustainable conservation (FAO 2006b). The suitable approach is important for a management strategy of indigenous AnGR (Figure 1.3).

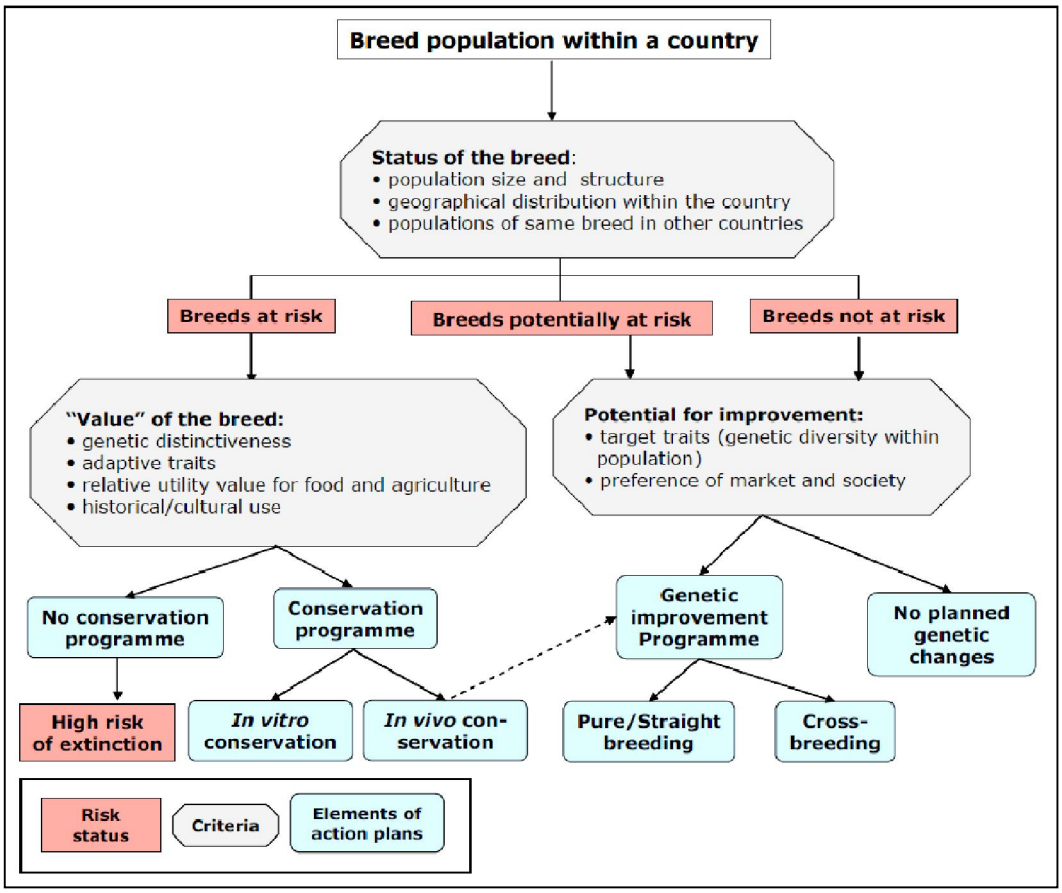


Figure 1.3 Design of animal genetic resources management strategies (FAO 2006b).

At present, however, the knowledge of the indigenous species is still limited and scattered among agencies. A further collaboration among the agencies within the country is required. The livestock sector is a system which combines all the components of biological diversity, the economy, social aspects and culture. The research purposes are sustainable livestock development in order to produce of the quality food as well as protect the safety of humans and the environments (Khumnirdpetch 2001; Khumnirdpetch 2002). Thus, research should emphasize the management of animal genetic resources as a part of the component of agricultural biodiversity. Breed improvement programmes have been initiated in some livestock species; dairy, beef, buffalo, and swine for a limited herds. A national breeding program is not available due to the lack of a recording system. *In vitro* conservation has been done through cryopreservation of the eggs, semen and embryo, collection of seeds, tissues and cells

and for forage, with microorganisms also being appropriate. *In vivo* conservation has been considered as a sustainable process and can have a large impact on community participation (FAO 2006b; 2007a; b). However, livestock production in Thailand is more commercialized. The question of what the efficient incentive measures for smallholders, producers and communities to participate in conservation are exists (Khumnirdpetch 2002).

The need to conserve and to utilize existing genetic diversity is a process where all stakeholders should participate for future benefits to mankind. Studies on genetics, the development of economic traits and the preservation of indigenous breeds are crucial to defining and registering genetic resources. Well planned breeding programmes and measures for effective communication, especially between the decision-makers, are urgently needed. Sustainable conservation of indigenous livestock genetic resource as a vital component within the agricultural biodiversity domain will be a great challenge, as well as a benefit for livestock production development of Thailand.

5 Objectives of the study

For the reason stated above, the major scope of this thesis is to describe the genetic information and background which is indispensable in order to conserve Thai pigs and cattle breeds as well as to putatively define them as genetic resources. In particular, this study is aimed at:

1. To investigate the mtDNA composition of indigenous pigs in Northern Thailand and to determine the genetic diversity.
2. To compare the genetic background of Thai indigenous pigs with commercial pigs used for meat production in Thailand, as well as to compare them with some Chinese pig breeds.
3. To assess the phylogeny of Thai indigenous pigs with further Asian and European pigs as well as to clarify their domestication origin.
4. To investigate the polymorphism of the bovine *HSP90AB1* genes.
5. To estimate physiological responses that are probably associated with heat tolerance traits in Thai native cattle breeds.

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CHAPTER 2

Genetic Structure and Variation in Thai Indigenous Pig Populations based on the mtDNA Control Region

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Genetic structure and variation in Thai indigenous pig populations based on the mtDNA control region

Abstract

Native pigs and wild boars are indigenous to Thailand. They are adapted well to hot and humid climates. It is difficult to determine specific phenotypic characteristics to distinguish the breeds and also very few studies have been conducted which investigate their mtDNA composition. Here we determined the genetic identity and diversity of them. The mtDNA control region of 72 Thai native pigs and 11 Thai wild boars indigenous to Northern Thailand was analysed by directed sequencing. In total 36 nucleotide variations leading to the formation of 24 different haplotypes were described (TNH01 to TNH02 and TWH01 to TWH04). Phylogenetics, molecular diversity and population structure of them were analyzed. The phylogenetic tree was separated into two main clades: a European (E) clade and an Asian (A) clade with further Asian subclades (AS1, AS2 and THG). Twenty-three of the 24 mtDNA haplotypes were integrated into the Asian clade of the phylogenetic tree and eight of them recapitulated another major cluster of haplotypes (THG). One haplotype (TNH01) fit to the European clade of the phylogenetic tree. The data implies that THG and AS1 diverged from the AS2 clade, but also that AS1 is evolutionarily older than THG. Moreover, this study suggested that Thai native pigs are closely related with Thai wild boars, but are also distinctly separated from them enough and can be traced back to the common Asian ancestor.

Keywords: Genetic variation, population structure, mtDNA, Thai pigs.

Introduction

Native pigs and wild boars are indigenous to Thailand whereas European pigs are regarded as commercial breeds in this country since they were imported in the 50's of the last century (Visitpanich and Falvey 1980). The domestic animal diversity information

system (DAD-IS; http://www.dad.fao.org) of the Food and Agriculture Organization (FAO) groups Thai native pigs (*Sus scrofa*) into four breeds Raad (Ka Done), Puang, Hailum (Hainan), and Kwai either by their physical appearances or with respect to the regions where they have been rampant. However, these breeds have been commonly bred for some 30 to 40 years. Thus, it is nowadays difficult to determine specific phenotypic characteristics to distinguish the breed lines and their origin also remains unknown due to poor documentation or even absence of records. Moreover, starting 20 to 30 years ago, many Thai native pigs have been increasingly mated with European commercial breeds (e.g. Large White, Duroc, and Hampshire) as well as with the Chinese Meishan to improve their performance for economically important traits. Consequently, most native pigs were gradually replaced by pure European pig breeds and have become even less suited to consumers' or breeding companies' needs. The number of native pigs has therefore steadily decreased. In 1994 less than 500 herdbook sows and less than 10 nucleus herds were registered in Thailand (Rattanaronchart 1994). Communities in the North/Northeast of the country predominantly keep Thai native pigs. Especially smallholders in the hill tribe communities traditionally raise a few indigenous pigs due to customs and religion. Animals are sacrificed on special celebrations such as New Year and weddings (Nakai 2008). However, small pig populations without any scrutinized breeding programs are always at the risk of losing genetic identity and diversity, as well as to becoming extinct. These are in agreement with a study which argues that pure native breeds are already on the edge of extinction (Rattanaronchart 1994).

The need to conserve and to utilize existing genetic diversity has become a concept of highest importance worldwide. Studies on genetics, the development of economic traits and the preservation of indigenous breeds are crucial to defining and registering genetic resources (Rege & Okeyo 2006). Molecular markers have proven to be the best tools available to estimate genetic diversity, to assess phylogenetic relationships and thus to ensure sustainable animal breeding. In pigs, microsatellites (e.g. Yang *et al.* 2003; Thuy *et al.* 2006), amplified fragment length polymorphism (AFLP) (e.g. Kim *et al.* 2002b) and mitochondrial DNA (mtDNA) sequence analysis (e.g. Watanobe *et al.* 1999) have been used. In particular, the mtDNA has been widely used being exclusively maternally inherited and highly polymorphic without any genetic recombination (Alves *et al.* 2003). The control region within the mtDNA (total length of the porcine mtDNA is 16,679

nucleotides; Ursing & Arnason 1998) has already been used to demonstrate genetic relationships between pig populations from Asia and Europe (e.g. Okumura *et al.* 2001; Fang & Anderson 2006; Wu *et al.* 2007a).

To our knowledge, very few studies have so far been conducted so far which investigate the mtDNA composition of Thai pigs. Here we determined the genetic identity and diversity of indigenous pigs in Northern Thailand.

Materials and Methods

Sample collection and DNA extraction

In this study DNA samples of 72 Thai native pigs (TNP) and 11 Thai wild boars (TWB) were used. Prior to mtDNA analysis, blood, ear clips or hair samples were collected on smallholder farms located in twelve localities/amphoe (an administrative subdivision of a province) in six Northern provinces, i.e. Chiang Rai, Chiang Mai, Mae Hongson, Lamphun, Nan and Uttaradit (Figure 2.1 and Table 2.1) between August 2005 and December 2006. Pigs were assigned to one of the five geographical sampling populations established according to the fauna and absolute distances based on GPS (Global Position System). The five region where the native pigs were chosen from, are the Mae Hongson region (MH), Southern Chiang Mai region (SCM), Northern Chiang Mai region (NCM), Chiang Rai region (CR) and Uttaradit region (UT). Thai wild boar samples (TWB) were assigned to one population, as they were originally caught in the wilderness of the two provinces Mae Hongson and Nan before kept in captivity.

Genomic DNA was extracted from blood and ear clips by a modified salting out method according to Sambrook *et al.* (1989) and Miller *et al.* (1988) or from hair roots using the QIAamp DNA Blood mini kit (Qiagen, Germany).

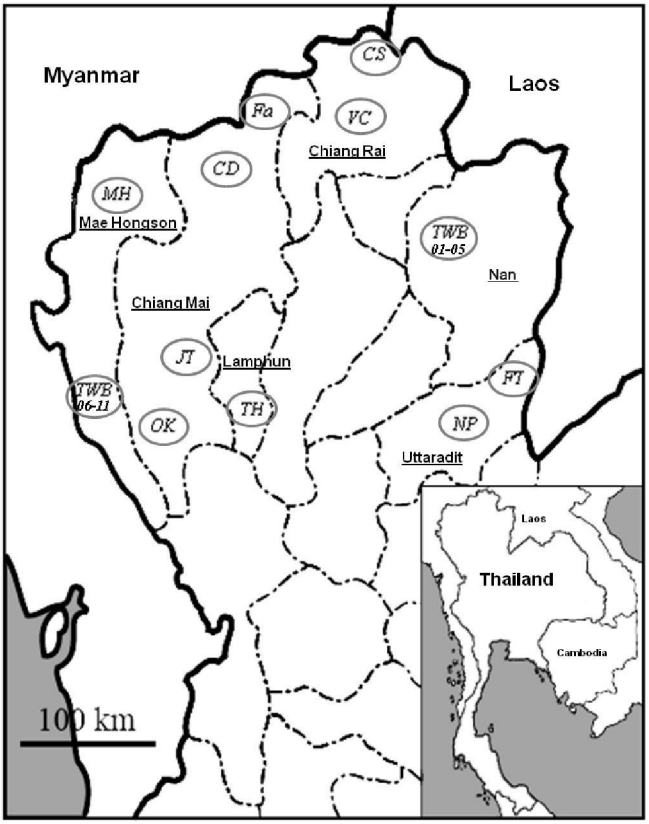

Figure 2.1 Origin and specification of the DNA samples. Underlined are the names of the provinces, indicated by circles are the localities (amphoe).

Table 2.1 Origin and specification of the DNA samples

Populations	Locality (Amphoe)	Coordinates (GPS data)*	Animal ID	n
MH	Muang (MH)	19° 16' 00" N, 097° 56' 00" E	MH01-06	6
SCM	Jhom Thong (JT)	18° 25' 37" N, 098° 40' 41" E	JT01-12	12
	Om Koi (OK)	17° 40' 00" N, 098° 25' 00" E	OK01-07	7
	Tung Huachang (TH)	18° 34' 52" N, 099° 00' 33" E	TH01	1
NCM	Fang (Fa)	19° 55' 00" N, 099° 13' 00" E	Fa01-04	4
	Chiang Dao (CD)	19° 22' 00" N, 098° 58' 00" E	CD01-03,10-16	10
CR	Chiang San (CS)	20° 16' 00" N, 100° 05' 00" E	CS01-08	8
	Viang Chai (VC)	19° 53' 00" N, 099° 55' 00" E	VC01-03	3
UT	Nam Pat (NP)	17° 35' 00" N, 100° 40' 00" E	NP01-11	11
	Fak Tha (FT)	18° 00' 00" N, 100° 55' 00" E	FT01-10	10
TWB	Ban Luang (TWB)	18° 51' 00" N, 100° 26' 18" E	TWB01-05	5
	Mae Sariang (TWB)	18° 10' 00" N, 098° 25' 00" E	TWB06-11	6

*GPS = Global Positioning system.

PCR amplification and sequencing of the mtDNA control region sequences

The control region (positions 15434 to 16679) of the mtDNA was amplified by PCR using PuReTaqTM Ready-To-GoTM PCR beads (Amersham Biosciences Europe, Germany). These freeze-dried beads contain 200 μM dNTP, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 2.5 units Taq polymerase and 1 x PCR buffer. Primers $DL4F^{15388}$ (5'-TCC ACC ATC AGC ACC CAA AG-3') located in the L-strand of the threonine tRNA and $DL4R^{37}$ (5'-TCC AGT GCC TTG CTT TAG TA-3') located in the H-strand of the phenylalanine tRNA were used to amplify a fragment comprising the entire mtDNA D-loop region. For direct DNA sequencing primer $DL4F^{15388}$ was tailed at the 5'-end with the universal M13 forward sequence and primer $DL4R^{37}$ with the universal M13 reverse sequence (Table 2.2). The PCR profile consisted of 35 cycles at 94 ºC for 1 min, an annealing temperature at 60 ºC for 1 min, and an extension period of 1 min at 72 ºC with an initial denaturation for 4 min at 94 ºC and a final extension at 72 ºC for 5 min. PCR reactions were performed on a Biometra T-Gradient thermocycler (Biometra, Germany). To check fragment integrity PCR products were separated on 1% agarose gels. PCR products were then purified with the QIAquick PCR Purification Kit (Qiagen, Germany).

Table 2.2 Sequencing primers

Primer	Sequence (5'-3')	T_m (ºC)
$M13^{uni}$	TGTAAAACGACGGCCAGT	53.7
$M13^{rev}$	CAGGAAACAGCTATGACC	53.7
$DLAF^{15758}$	TACCATGCCGCGTGAAACCA	59.4
$DL1R^{15900}$	TGGGCGATTTTAGGTGAGAT	55.3
$DL2F^{16088}$	ACGACAATCCAAACAAGGTG	55.3
$DL3R^{16387}$	GGGGGTTTGAATGAGCTAATAA	56.5
$DL5F^{16346}$	CGCGCATATAAGCAGGTAAA	55.3
$DL6R^{16147}$	CGTGCATATAAGCAGGTAAA	59.4

The purified PCR products were directly sequenced using the BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit® (Applied Biosystems, Germany) on an automated DNA sequencer (ABI-PRISM 3100® capillary analyzer; Applied Biosystems, Germany). Eight sequencing primers were used to generate double-stranded overlapping contigs covering the whole D-loop region (Figure S1; Table S1). The sequenced data were analyzed and manually checked using the software suite DNASTAR LasergeneTM 6® (DNASTAR, Inc., Germany).

Data analyses

Sequence alignments were performed using the ClustalW (v. 1.83) multiple sequence alignment program (Chenna *et al*. 2003). The highly variable tandem repeat 5'-CGTGCGTACA-3' and the 11 bp indel 5'-TAAAACACTTA-3' (see also Results and discussion) were excluded (Kim *et al*. 2002a; Wu *et al*. 2007a) from the analysis. Alignments were checked manually and edited in any format using the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.* 2007).

Population indices were calculated using the ARLEQUIN program version 3.0 (Excoffier *et al.* 2005). To estimate the genetic diversity and the genetic structure, the nucleotide diversity (Tamura & Nei 1993) and the haplotype diversity (Nei 1987) model of sequence evolution were calculated for each pig population. Corrected average pairwise differences (PiA) between pig groups were calculated using the equation PiA= PiXY - (PiX+PiY)/2, where PiXY is the population average pairwise nucleotide difference within pig groups X and Y (Nei 1987). The significance of differences between pig groups was tested using 10,00 permutation steps in ARLEQUIN.

To characterize the relationships of mtDNA haplotypes, median-joining (MJ) networks were constructed following the algorithms of Bandelt *et al.* (1995) to eliminate non-parsimonious links and to establish a simpler network structure. All variable characters of complete alignment were entered into the software package NETWORK 4.5 (Bandelt *et al.* 1999). Frequencies of haplotypes were converted into proportional areas in the figures. Phylogenetic analyses were performed using the maximum likelihood (ML) and the neighbor-joining (NJ) method. Prior to that the best-fitting model of evolution was identified by MODELTEST version 3.7 (Posada &

Crandall 1998). The Tamura-Nei (TrN) model assuming a proportion of invariant sites (*I*) and a gamma distribution for rate variation among sites (*G*) was selected by MODELTEST (*I* = 0.77; *G* = 0.73). ML analyses were carried out with the software PAUP4.0b10 (Swafford 2002). Genetic distances based on the gamma distribution (*G* = 0.73) using the Tamura-Nei algorithm (Tamura & Nei 1993) were implemented in the MEGA software. NJ trees (Saito & Nei 1987) were simulated based on the estimated distance matrix. Finally, the bootstrap method (Felsenstein 1985) was used to determine the respective confidence interval with 10,000 bootstrap repetitions for the NJ tree.

The phylogenetic analysis of the Thai pigs included further 60 mtDNA D-loop sequences (Table S2.1; Okumura *et al.* (1996), Groves *et al.* (1997), Kim *et al.* (2002a), Watanobe *et al.* (2002), Gongora *et al.* (2004), Wu *et al.* (2007a), and Wu *et al.* (2007b)). These mtDNA D-loop sequences have previously been published or have alternatively been deposited in GenBank without a relevant publication. The resulting phylogenetic tree includes all currently available mtDNA sequences of pigs indigenous to (South-Eastern) Asian countries: 22 Chinese, four Taiwanese, two Korean, two Japanese, one Vietnamese, eight European domestic, and two Göttingen miniature pigs as well as six feral pigs indigenous to Australia and New Zealand. Ten Asian and three European wild boars were also considered (Table S2.1).

Time since divergence (T) was estimated according to Li (1997) using the sequence divergence (d) and the substitution rate (r) and the equation: T = d/2r. A maximum substitution rate of 7.5×10^{-8} per site and year as assessed in humans (Tamura & Nei 1993) and a minimum rate of 1.37×10^{-8} per site and year as assessed in mammals (Pesole *et al.* 1999) were assumed.

Results and Discussion

Sequence evaluation

Eight primers per fragment were used to sequence the whole control region double stranded. Sequences were compared with the reference sequence AJ002189 (Ursing & Arnason 1998; Figure 2.2). Comparative sequencing of the 83 samples revealed individual fragment length differences. PCR fragment lengths ranged between 1264 bp

and 1324 bp. These deviations were due to either the number of the porcine heteroplasmic (Ghivizzani *et al.* 1993; Lunt *et al.* 1998) 10-bp repeat motif CGTGCGTACA (stretching from position 15434 to 16679 of the porcine mtDNA reference sequence AJ002189; Ursing & Arnason 1998) or to a minor degree to the existence of indels in the respective samples. The observed numbers of repeat units (22 to 28) were randomly and irrespectively of the animals' geographic origins. This remarkable numeric variability of the repeat unit is in strong agreement with investigations conducted by others. Ghivizzani *et al.* (1993) described in their sampling a minimum of 14 and a maximum of 29 repeat units. All repeat motifs except one were finally removed prior to the phylogenetic studies despite that a considerable potential of mtVNTRs (mitochondrial variable number of tandem repeats) has been postulated to assess genetic diversity or phylogenetic relationships (Lunt *et al.* 1998).

In total 36 nucleotide polymorphisms were found in the 83 investigated animals accounting for 24 different mtDNA haplotypes termed TNH01 to TNH20 and TWH01 to TWH04. TNH01 finally proved to be the haplotype with the least sequence variations (in total 2), whereas the number of variations varied in the further 23 haplotypes from 14 (TNH20) to 24 (TNH11) compared to the GenBank reference sequence. The vast majority (21 out of 24) of haplotypes turned out to be novel, but eight of them were also animal-specific (TNH02, TNH05, TNH07, TNH08, TNH09, TNH12, TNH17 and TWH02). Complete matches between TNH16 and one Göttingen miniature pig haplotype (AY463067) existed. The haplotype specific for the Okinawa (AB015092), the Wanan (AF276924) and the Taoyuan pig (GQ169775) is identical to TWH02. Haplotype TWH04 corresponds to the second known mtDNA sequence of a Göttingen miniature pig (AY463068) also described for the Satsuma pig (AB015091). The 36 polymorphic sites represent 3.45 % of the analyzed DNA sequence (1044 bp). There was a strong bias towards transitions instead of transversions: in contrast to 33 transitions (16 A↔G and 17 C↔T), no transversions could be detected. Prior to us others have also reported the strong prevalence for transitions in the porcine mtDNA (Kim *et al.* 2002a; Gongora *et al.* 2004). The transitition to transversion ratio is a well-described pattern of primate mtDNA sequence evolution (Kocher *et al.* 1989). In very close relatives (e.g. a species within a genus) most of the changes are transitions, whereas transversions are more evident among more distant relatives (e.g. a genera within a family).

Haplotype	Animals ID	Polymorphic sites
		2 4 / 1 0 9 / 1 2 3 / 1 2 8 / 1 3 6 / 1 4 9 / 1 4 4 / 1 5 9 / 1 5 4 / 1 8 2 / 1 8 3 / 2 1 5 / 2 2 4 / 2 4 7 / 2 7 9 / 2 8 1 / 2 9 6 / 3 0 2 / 3 0 7 / 3 2 8 / 3 3 5 / 3 4 9 / 3 9 2 / 4 0 7 / 4 4 5 / 4 5 4 / 5 0 3 / 5 6 2 / 6 7 7 / 7 9 4 / 9 0 6 / 8 9 6 / 9 6 1 / 9 6 2 / 9 9 9 / 1 0 1 9
	AJ002189	G T - G C C A C A T T T T C A T A C A C T A C T A C A T A A G A A T A T
TNH01	NP01,03, FT01,02,07	. . . . . - . C . . . . . G . . . .
TNH02	NP08	. C A A - T . T G C . . . - . C G . . T . . . G T G . G G . . . C G C
TNH03	CS06,07, NP09	A C . A - T . T G C C C C - . C G . . T . . T . G T G . G G . G . C G C
TNH04	FT04,06	A C . A - T . T G C C C . - . C G T . T . . T . . . G C G G . G . C G C
TNH05	OK03	. C . A - T . T G C . . C - . . G . . T . . T . . T . . G G A . . C G C
TNH06	OK01,02,04,05,07	. C . A - T . T G C . . C - . C G . . T . . T . . T . C G G A . . C G C
TNH07	FT08	A C . A - T . T G C C C . - . C G . . T . . T . . . G C G G . G . C G C
TNH08	NP10	A C . A - T . T G C C . C - . C G . . T . . T . . T G C G . . G . C G C
TNH09	FT03	. C . A - T . T G C . . . - . C G . . T . . T . . . . . G G . . . C G C
TNH10	CS01,05, JT01,04,05,09	. C . A - T . T G C . . C - . . G . . T . . T . . . . C G G . . G C G C
TNH11	CS02, NP02,04-07,11, FT05,09	A C . A - T . T G C C C C - . C G . . T . . T . . . G C G G . G . C G C
TNH12	CS08	A C . A - T . T G C C C C - . C G . . T . . T . . T . . G G . G . C G C
TNH13	Fa01-04	. C . A - T . T G C . . C - . . G . . T . . T . . T . . G G . . . C G C
TNH14	JT02,03	. C . A - T . T G C . . . - . . G . . T C . T . . . G . G G . . . C G C
TNH15	JT06,07	. C . A - T . T G C . . C - . C G . . T . . T . G T . . G G . . . C G C
TNH16	JT08,10,12	. C . A - T . T G C . . C - . C G . . T . . T . . T . . G G . . . C G C
TNH17	JT11	. C . A - T . T G C . . . - . C G . . T . . T . G T G . G G . . . C G C
TNH18	CS03,04 VC01-03, TH01	A C . A - T . T G C C C C - . C G . . T . G T . . . G . G G . G . C G C
TNH19	MH01-03,05	. C . A - T G T G . . . . - . . G . G T . . . . . C G G . . . C G C
TNH20	CD01-03,10-15, OK06, MH04,06, FT10	. C . A - T . T G C . . . - . . G . . T . . T . . . C G . . . . C G C
TWH01	TWB06,09	. C . A - T . T G C . . . - . C G . . T . . T . G T G . G . . . . C . C
TWH02	TWB03	. C . A - T . T G C . . . - . . G . . T . . T . . . . . G G . . . C G C
TWH03	TWB01,02,04,05	A C . A - T . T G C C C . - G C G . . T . . T . . . G . G G . G . C G C
TWH04	TWB7,8,10,11	. C . A - T . T G C . . C - . C G . . T . . T C . T . . G G . . . C G C

Figure 2.2 Characterization of the 24 Thai haplotypes and their variable positions. Dots (.) indicate matches with the reference sequence AJ002189, minus (-) indicates indels or gaps. The 11-bp indel specific for haplotype TWH04 (nucleotides 899 to 909) is not shown.

An 11-bp long indel (5'-TAAAACACTTA-3') at positions 899 to 909 confirmed observations by others who detected this sequence variation also in Asian (Okumura *et al.* 2001 and Wu *et al*. 2007a) as well as European and American pig breeds (Okumura *et al.* 2001). The resulting haplotype TWH04 was specific for Thai wild boars caught in the amphur Mae Sariang (i.e. TWB 7, 8, 10 and 11), but was not common for all pigs representing this sub-sampling. In addition, three one-bp indels were found at positions 121, 138, and 274 each. Insertion A at position 121 and deletion C at position 274 were common for all Thai haplotypes, whereas the one-bp indel at position 138 (position 137 in Okumura *et al.* 2001) was exclusively present in haplotype TNH01 with a total of 5 observations in our DNA repository.

Haplotype distribution and population structure

The distribution of haplotypes and the absolute numbers of individuals per haplotype and sampling population are shown in Table 2.3. Twenty haplotypes TNH01 to TNH20

were specific for Thai native pigs, and four haplotypes (TWH01 to TWH04) were specific for Thai wild boars. Considering both the number of investigated animals and the number of haplotypes per population an enhanced genetic diversity for Thai native pigs in SCM, CR and CT is evident. By contrast only five (TNH03, TNH10, TNH11, TNH18, and TNH20) of the 20 described haplotypes were observed in more than one sampling population: TNH03 and TNH11 in CR and UT, TNH10 and TNH18 in SCM and CR. Pigs with TNH20 belonged to four sampling populations I, II, III and V, but were predominantly found in sampling population III. The local distribution of these five haplotypes proposes a putative enhanced genetic exchange between animals in the past or might be the consequence of frequent transportation today. A MJ network considering the 24 haplotypes was constructed (Figure 2.3). The least diverse haplotype TNH01 could not be integrated into any of the Thai haplotype and revealed instead the highest genetic distance to the other. The four Thai wild boar haplotypes proved to be genetically distinct, were not grouped together, but became part of the haplogroups. The investigated wild boars were kept as separate groups and housed in pens by the smallholders at the time of the sampling. They were also not mated with Thai native pigs, but occasional matting in the past cannot be ruled out.

We further evaluated the molecular diversity and determined the genetic structure of the investigated Thai pig populations by population statistics (Table 2.4). The haplotype diversity index (*H*) varied between 0.4396 (NCM) and 0.8895 (SCM), reflecting the low number of haplotypes distribution (*f*) in NCM ($f = 2$) and the high number of observed haplotypes in SCM ($f = 9$). The haplotype diversity of 0.7636 for Thai wild boars is similar to the indices calculated for CR and UT animals, but lower than the overall haplotype diversity ($H = 0.9331$) of Thai native pigs (irrespective of the sampling population). High haplotype diversities were not supported by nucleotide diversity (π) estimates (e.g. SCM). Thai wild boars revealed the highest - putatively a result of extensive migration - nucleotide diversity ($\pi = 0.0058$), which was not reflected by the haplotype diversity index either. Nucleotide diversity proved over all to be low in Thai pigs.

Table 2.3 Haplotypes and number of individuals per haplotype at the sampling populations

Haplotypes[1]	MH	SCM			NCM		CR		UT		TWB		Total
	MH	JT	OK	TH	Fa	CD	CS	VC	NP	FT	01-05	06-11	
TNH01	-	-	-	-	-	-	-	-	2	3	-	-	5
TNH02	-	-	-	-	-	-	-	-	1	-	-	-	1
TNH03	-	-	-	-	-	-	2	-	1	-	-	-	3
TNH04	-	-	-	-	-	-	-	-	-	2	-	-	2
TNH05	-	-	1	-	-	-	-	-	-	-	-	-	1
TNH06	-	-	5	-	-	-	-	-	-	-	-	-	5
TNH07	-	-	-	-	-	-	-	-	-	1	-	-	1
TNH08	-	-	-	-	-	-	-	-	1	-	-	-	1
TNH09	-	-	-	-	-	-	-	-	-	1	-	-	1
TNH10	-	4	-	-	-	-	2	-	-	-	-	-	6
TNH11	-	-	-	-	-	-	1	-	6	2	-	-	9
TNH12	-	-	-	-	-	-	1	-	-	-	-	-	1
TNH13	-	-	-	-	4	-	-	-	-	-	-	-	4
TNH14	-	2	-	-	-	-	-	-	-	-	-	-	2
TNH15	-	2	-	-	-	-	-	-	-	-	-	-	2
TNH16	-	3	-	-	-	-	-	-	-	-	-	-	3
TNH17	-	1	-	-	-	-	-	-	-	-	-	-	1
TNH18	-	-	-	1	-	-	2	3	-	-	-	-	6
TNH19	4	-	-	-	-	-	-	-	-	-	-	-	4
TNH20	2	-	1	-	-	10	-	-	-	1	-	-	14
TWH01	-	-	-	-	-	-	-	-	-	-	-	2	2
TWH02	-	-	-	-	-	-	-	-	-	-	1	-	1
TWH03	-	-	-	-	-	-	-	-	-	-	4	-	4
TWH04	-	-	-	-	-	-	-	-	-	-	-	4	4
Individuals/ Haplotpyes	6/2	20/9			14/2		11/5		21/9		11/4		83/24

[1] TNH = Thai native pig haplotypes, TWH = Thai wild boar haplotypes.

Table 2.4 Molecular diversity of Thai native pigs and Thai wild boars[1]

Populations	n	*f*	π	H
MH	6	2	0.0026 ± 0.0018	0.5333 ± 0.1721
SCM	20	9	0.0043 ± 0.0025	0.8895 ± 0.0416
NCM	14	2	0.0017 ± 0.0012	0.4396 ± 0.1120
CR	11	5	0.0041 ± 0.0012	0.7818 ± 0.1073
UT	21	9	0.0035 ± 0.0021	0.7583 ± 0.1104
TWB	11	4	0.0058 ± 0.0034	0.7636 ± 0.0833

[1] n = sample size, *f* = haplotype distribution, π = nucleotide diversity (± SE), H = haplotype diversity (± SE).

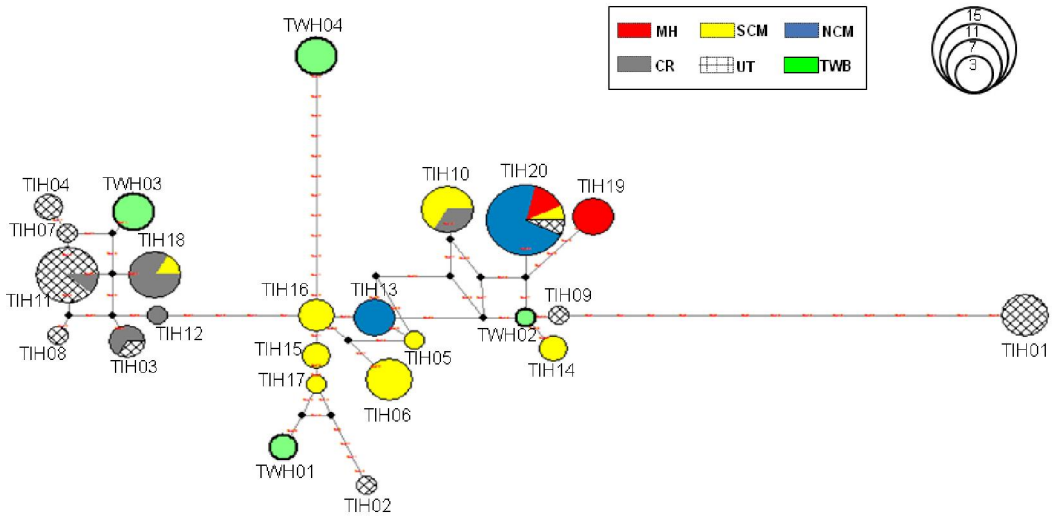

Figure 2.3 Median-joining network profile. The circle size corresponds to the haplotype frequency. If animals of one population shared more than one haplotype, the pie illustrates the respective proportions. The Median vector (mv), produced by the network software, representing missing or not sampled haplotypes is illustrated by small solid dots.

The average of pairwise genetic differences (Table 2.5) within populations (Pi_X) supports the limited genetic diversity of Thai pigs located in NCM (1.8287). The highest genetic diversity of Thai native pigs was 4.6135 in SCM, but clearly lower than the one calculated for Thai wild boars (6.2072). Average pairwise differences between populations (Pi_{XY}) ranged from 4.2397 to 11.5233 and all populations were significantly different ($p \leq 0.05$). Pigs deriving from MH showed the highest pairwise differences to animals located in the further sampling locations (SCM 7.2132, NCM 4.2993, CR 11.5233, UT 9.9309, and TWB 9.6530).

Phylogenetics

The domestic pig originates from the Eurasian wild boar (Giuffra *et al.* 2000), but domestication itself occurred at different centres in SEA (Larson *et al.* 2005). This article on the phylogeography of wild boars and a follow-up on the Neolithic expansion in ISEA (Island South East Asia) and Oceania (Larson *et al.* 2007) include only four porcine specimens from Thailand provided by some museums. Unfortunately, the locations of the samples were not available in the respective museum records (Larson *et al.* 2005).

Table 2.5 Population pairwise genetic differences[1]

Populations	MH	SCM	NCM	CR	UT	TWB
MH	**2.7913**	7.2132*	4.2993*	11.5233*	9.9309*	9.6530*
SCM	3.5107*	**4.6135**	4.2397*	7.6888*	7.5368*	6.6270*
NCM	1.9893*	1.0186*	**1.8287**	8.6148*	7.7378*	6.7001*
CR	7.9325*	3.1869*	5.5053*	**4.3902**	4.5412*	6.9194*
UT	6.6365*	3.3313*	4.9247*	0.4473*	**3.7974**	6.7706*
TWB	5.1537*	1.2166*	2.6821*	1.6206*	1.7683*	**6.2072**

[1] Above diagonal: average number of pairwise differences between populations (Pi_{XY}); diagonal elements (in bold): average number of pairwise differences within population (Pi_X); below diagonal: corrected average pairwise difference ($Pi_{XY} - (Pi_X+Pi_Y)/2$). Asterisks indicate significant differences ($p< 0.05$).

In this present study, the phylogenetic tree refers to 1044 bp of mtDNA sequence. The applied model for this data set was: TrN + *I* + *G* with an estimated shape parameter for the gamma distribution of 0.73 and an estimated proportion of variable sites of 0.77. Phylogenetic trees calculated according to the NJ method. The tree separates two main clades, i.e. a Europe (E) and an Asia (AS) clade with further Asian subclades, an observation also reported by others before (e.g. Okumura *et al.* 2001; Wu *et al.* 2007a; Wu *et al.* 2007b). The constituted Asian clade divides further into two larger subclades (AS1 and AS2) and a single haplotype representing the Taiwanese Lanyu pig that finally could not be assigned to either one of the two Asian subclades. Subclade AS2 contains sequences of the Japanese Ryukyu wild boar, the Korean wild boar and the Indochinese warty pig indigenous to Laos. Twenty-three of the twenty-four haplotypes arranged randomly in AS1 that consists of 71 haplotypes including Asian domestic pigs, Asian wild boars, feral pigs from Australia and New Zealand but also the Göttingen miniature pig. The subclade AS1 corresponds to the D2 cluster, which is distributed widely in Chinese domestic pigs and global pig breeds that have some relationships with Asian pigs, as well as the East Asian wild boars (Larson *et al.* 2005). The only Thai haplotype that was not arranged to subclade AS1, but to the European clade was TNH01. Despite the phenotypic differences, genetic exchanges of Thai pigs with exotic maternal lineages have been possible. The Department of Livestock Development (DLD) at the Ministry of Agriculture began to import European pig breeds into Thailand in 1957 and has promoted the raising and selling of them in local areas (Rattanaronchart 1994).

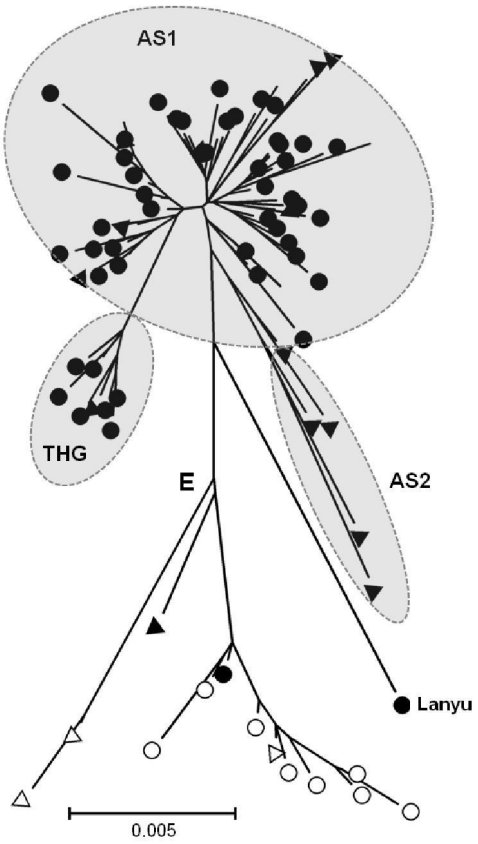

Figure 2.4 Phylogenetic tree of Thai, European and further Asian pigs based on 84 mtDNA sequences representing the Asian domestic pigs (black circle), Asian wild boars (black triangle), European wild boars (white triangle) and European domestic pigs (white circle). The tree was constructed by the neighbour-joining method and is shown in the radiation form.

Eight of the 23 Thai haplotypes recapitulated another major cluster supported by a high bootstrap value (77%) which was denoted as the Thai haplogroup (THG). Average pairwise distances of 0.0136 ± 0.0029 (between AS2 and THG), of 0.0109 ± 0.0023 (between AS2 and AS1) and of 0.0084 ± 0.0023 (between THG and AS1) resulted in estimates for the time since divergence of 90,000 to 496,000 years between mtDNA clade AS2 and clade THG, 72,000 to 397,000 years between clade AS2 and clade AS1, and 56,000 to 306,000 years between clade THG and clade AS1. The data imply that THG and AS1 diverged from the AS2 clade, but also that AS1 is evolutionary older than THG. The data support even more the hypothesis that anchestoral animals of both AS2 and AS1 contributed to establish THG. It is likely that

the indigenous Thai pigs reported within subclade AS1 have the same origin as Chinese domestic pigs (e.g. Larson *et al.* 2005; Fang & Anderson 2006; Tanaka *et al.* 2008). THG haplotypes are, however, most putatively the consequence of another independent domestication event in SEA as no Chinese pigs are arranged within this haplogroup.

Our present study showed that Thai native pigs are closely related with Thai wild boars, but are also distinctly separated enough from them and eventually can be traced to the common Asian ancestor. Modern animal breeding depends on the characterization and classification of breeds. That requires both mtDNA analysis to determine their maternal ancestors and also nuclear DNA profiling to understand their present genetics. The provided genetic information is therefore a benefit for both conservation purposes as well as the utilization of them as an important genetic resource in the field of disease resistance as well.

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Supplementary material

Table S2.1 Publicly available Porcine mtDNA sequences for phylogenetic analysis

No.	Code	Breeds	Accession numbers	Type
1	IWB-I	Italian wild boar I	AB015094	European wild boar (Italy)
2	IWB-II	Italian wild boar II	AB015095	European wild boar (Italy)
3	EWB	European wild boar	FJ237000	European wild boar
4	ICW	Indochinese warty pig	DQ444703	Asian wild boar (Laos)
5	VWB	Vietnamese wild boar	EF545584	Asian wild boar (Vietnam)
6	CWB-I	Chinese wild boar I	EU333163	Asian wild boar (China)
7	CWB-II	Chinese wild boar II	EF545569	Asian wild boar (China)
8	RWB-I	Ryukyu wild boar I	AB015087	Asian wild boar (Japan)
9	RWB-II	Ryukyu wild boar II	AB015090	Asian wild boar (Japan)
10	JWB-I	Japanese wild boar I	AB015085	Asian wild boar (Japan)
11	JWB-II	Japanese wild boar II	AB015084	Asian wild boar (Japan)
12	KWB-I	Korean wild boar I	AY574047	Asian wild boar (Korea)
13	KWB-II	Korean wild boar II	EF533685	Asian wild boar (Korea)
14	SW	Swedish commercial	AJ002189	European pig
15	HS	Hampshire	AB041488	European pig
16	LW	Large White	AB041492	European pig
17	PT	Pietrain	AB041489	European pig
18	DR	Duroc	AB041486	European pig
19	LR	Landrace	AB041496	European pig
20	BS	Berkshire	AB041484	European pig
21	IB	Iberian	EU117375	European pig
22	KK	Kune Kune	AY463076	New Zealand feral pig
23	OB	Oberon	AY463088	Australian feral pig
24	WT	Westran	AF276921	Australian feral pig
25	JC	Julia Creek	AY463092	Australian feral pig
26	VR	Vanrook	AY463094	Australian feral pig
27	ML	Mount Larcom	AY463093	Australian feral pig
28	GM-I	Goettingen miniature I	AY463067	Goettingen mini pig
29	GM-II	Goettingen miniature II	AY463068	Goettingen mini pig
30	CJ	Che Ju	AF276933	Asian pig (Korea)
31	JJ	Jeju	DQ191229	Asian pig (Korea)
32	JH	Jinhua	AB041475	Asian pig (China)
33	TC	Tong Cheng	AF276923	Asian pig (China)
34	MS-I	Meishan I	D17739	Asian pig (China)
35	MS-II	Meishan II	GQ169776	Asian pig (China)
36	WA	Wanan	AF276924	Asian pig (China)
37	WH	Wanhua	AF276932	Asian pig (China)
38	WN	Wannanhua	AF276925	Asian pig (China)
39	YX	Yanxin	AF276927	Asian pig (China)
40	GS-I	Ganzhongnan Spotted I	AY463061	Asian pig (China)

Table S2.1 (continue)

No.	Code	Breeds	Accession numbers	Type
41	GS-II	Ganzhongnan Spotted II	AY486115	Asian pig (China)
42	PU	Putian	AF276931	Asian pig (China)
43	TB	Tibetan	AY486116	Asian pig (China)
44	GX	Guan Xiang	AY486117	Asian pig (China)
45	GD	Gandonghei	AF276928	Asian pig (China)
46	NJ	Neijang	AF276929	Asian pig (China)
47	GZ	Guizhou Xiang	AY486118	Asian pig (China)
48	EL	Erhualian	AF276922	Asian pig (China)
49	HZ	Huzu	EF545588	Asian pig (China)
50	JG	Jinghua	AF276930	Asian pig (China)
51	QP	Qingping	EF545581	Asian pig (China)
52	YS	Yunnan Saba	EF545567	Asian pig (China)
53	SA	Sichuan Aba	EF545578	Asian pig (China)
54	LY-I	Lanyu I	EF375877	Asian pig (Taiwan)
55	LY-II	Lanyu II	DQ972936	Asian pig (Taiwan)
56	TY-I	Taoyuan I	AM040645	Asian pig (Taiwan)
57	TY-II	Taoyuan II	GQ169775	Asian pig (Taiwan)
58	SM	Satsuma	AB015091	Asian pig (Japan)
59	OW	Okinawa	AB015092	Asian pig (Japan)
60	MC	Mon Cai	AB041481	Asian pig (Vietnam)

*Okumura *et al.* (1996), Groves *et al.* (1997), Kim *et al.* (2002), Watanobe *et al.* (1999), Gongora *et al.* (2004), Wu *et al.* (2007a), and Wu *et al.* (2007b).

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CHAPTER 3

Further Resolution of Porcine Phylogeny in Southeast Asia by Thai mtDNA Haplotypes

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Further resolution of porcine phylogeny in Southeast Asia by Thai mtDNA haplotypes

Abstract

The mtDNA control region of 72 Thai native pigs and 11 Thai wild boars was comparatively sequenced. In total, 36 nucleotide variations that accounted for 24 haplotypes have been described (TNH01 to TNH20 and TWH01 to TWH04). These haplotypes and further publicly available mtDNA haplotypes were used to assess phylogenetic relationships. Twenty-three of the 24 haplotypes became integrated into the Asian clade of the phylogenetic tree and eight of them recapitulated another major cluster of haplotypes within this clade (Thai haplogroup, THG). Only haplotype TNH01 fit in with the European clade of the phylogenetic tree. An additional analysis using 510 bp of the mtDNA incorporated the THG haplotypes in to clade MTSEA (mountainous and Southeast Asian distribution) to form haplogroup MTSEA-THG. Recently, MTSEA was renamed in MC3. MC3 contains only signatures of pigs scattered across the Indo-Burma Biodiversity Hotspot (IBBH), a region including Thailand to the Kra Isthmus. Here we propose a putative independent porcine domestication event in South-east Asia (SEA). All haplotypes of haplogroup MTSEA-THG have revealed unique and previously unknown nucleotide signatures at positions 24 (nucleotide A) and at positions 183 (nucleotide C) that differentiate them from all other porcine mtDNA haplotypes.

Keywords: mtDNA, phylogeny, Thai indigenous pigs.

The domestic animal diversity information system (DAD-IS; http://www.dad.fao.org) of the Food and Agriculture Organization (FAO) has listed four Thai native pig breeds: Raad, Puang, Hailum, and Kwai. The individual assignment depends basically on regions where the animals have been rampant. Alternatively, the physical appearance is considered as an indicator although it is hardly possible to attribute individually specific

phenotypic characteristics after being commonly raised for several decades (Rattanaronchart 1994). Most of the Thai pigs are pigmented and are also well adapted to hot and humid climates. They are mainly kept without any vaccination and with low quality foodstuff. Our mtDNA analyses aimed thus to gain genetic information that is indispensable to conserve Thai breeds and to putatively define them as genetic resources. In addition the degree of phylogenetic relationships with further Asian and European pig breeds should be assessed.

To do so we collected samples of 72 Thai native pigs (TNP) and 11 Thai wild boars (TWB) in six Northern provinces or localities/Amphoe of the country (Table 3.1). Each TNP was assigned to one of five geographical sampling populations (Thai native pig populations, TNPP) established according to the predominant fauna and the absolute distances based on GPS (Global Position System) data. Thai wild boars (TWB) were regarded as one population, as they were originally caught in the wilderness before kept in captivity. We sequenced positions 15434 to 16679 of the control region (primers DL4F15388 and DL4R37). The details of primers and PCR conditions are presented in Chapter 2. The primer walking method, an ABI-PRISM 3100® capillary analyzer and the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit® (Applied Biosystems) were applied to gain bi-directionally sequence information. Prior to bioinformatics, sequence reads were manually scored using the software suite DNASTAR LasergeneTM 6® (DNASTAR, Inc.) and were finally aligned using ClustalW (v. 1.83) (Chenna et al. 2003).

A rare 11-bp indel 5'-TAAAACACTTA-3' was excluded from the data pool. Moreover, only one copy of the tandem repeat 5'-CGTGCGTACA-3' (Ghivizzani *et al.* 1993; positions 15434 to 16679 of the reference sequence AJ002189; Ursing & Arnason 1998) was incorporated in the analyses (Kim et al. 2002; Wu et al. 2007a; b). We observed individual fragment length differences of 1264 bp to 1324 bp for the investigated samples based on either the predominant number of the mtVNTR or of the indels. In total 36 nucleotide polymorphisms that accounted for 24 different mtDNA haplotypes were detected (TNH01 to TNH20 and TWH01 to TWH04). The distribution of haplotypes and the absolute numbers of individuals per haplotype are shown in Table 3.1. The polymorphic sites are equal to 3.45 % of the analyzed DNA sequence (1044 bp). A strong bias towards transitions instead of transversions (33 transitions (16 A↔G

and 17 C↔T)) was observed as reported by others before (Kim *et al.* 2002; Gongora *et al.* 2004). Three one-bp indels existed at positions 121, 138, and 274 each. The insertion A at position 121 and the deletion C at position 274 were common for all Thai haplotypes, whereas the indel at position 138 (position 137 in Okumura *et al.* 2001) was exclusively present in haplotype TNH01 with a total of 5 observations in our DNA repository.

Table 3.1 Origin of research populations and haplotype distribution[1]

Population	Province	Locality/Amphur	No. of individuals/ No. of haplotypes	Haplotype IDs (No. of observation)
TNP	Mae Hongson	Muang (MH)	6/2	TNH19 (4), TNH20 (2)
	Chiang Mai	Jhom Thong (JT)	12/5	TNH10 (4), TNH14 (2), TNH15 (2), TNH16 (3), TNH17 (1)
		Om Koi (OK)	7/3	TNH05 (1), TNH06 (5), TNH20 (1)
	Lamphun	Tung Huachang (TH)	1/1	TNH18 (1)
	Chiang Mai	Fang (Fa)	4/1	TNH19 (4)
		Chiang Dao (CD)	10/1	TNH20 (10)
	Chiang Rai	Chiang San (CS)	8/5	TNH03 (2), TNH10 (2), TNH11 (1), TNH12(1), TNH18 (2)
		Viang Chai (VC)	3/1	TNH18 (3)
	Uttaradit	Nam Pat (NP)	11/5	TNH01 (2), TNH02 (1), TNH03 (1), TNH08 (1), TNH11 (6)
		Fak Tha (FT)	10/6	TNH01 (3), TNH04 (2), TNH07 (1), TNH09 (1), TNH11 (2), TNH20 (1)
TWB	Nan	Ban Luang (TWB01-05)	5/2	TWH02 (1), TWH03 (4)
	Mae Hongson	Mae Sariang (TWB06-11)	6/2	TWH01 (2), TWH04 (4)

[1] See also Figure 2.1

The domestic pig originates from the Eurasian wild boar (Giuffra *et al.* 2000), and it is also well described that domestication occurred at different centres in ISEA (Island South East Asia) (Larson *et al.* 2005). That paper and a further one on the Neolithic expansion in ISEA and Oceania (Larson *et al.* 2007) included only four porcine specimens that derived from Thailand. The precise locations of the samples were

unfortunately not available in the respective museum records (Larson *et al.* 2005). To perform phylogenetic analyses for the here described haplotypes we applied both the maximum likelihood (ML) and the neighbor-joining (NJ) method and implemented in addition 60 publicly available mtDNA sequences of further pig sources. Included in the analysis were all known haplotypes of pigs indigenous to (South-East) Asian countries (Table S3.1). Prior to that the best-fitting Tamura-Nei (TrN) model assuming a proportion of invariant sites (I = 0.77) and a gamma distribution for rate variation among sites (G = 0.73) was identified by MODELTEST version 3.7 (Posada & Crandall 1998). ML analyses were carried out with PAUP4.0b10 (Swafford 2002). Genetic distances using the Tamura-Nei algorithm (Tamura & Nei 1993) were implemented in the MEGA software. NJ consensus trees (Saito & Nei 1987) were simulated based on the estimated distance matrix. 10,000 bootstrap repetitions (Felsenstein 1985) determined the confidence intervals of the phylogentic trees. The phylogenetic tree according to the NJ method (Figure 3.1) revealed an improved resolution and provided the same classification as the one using the ML algorithms (Figure S3.1).

The NJ tree has separated into two main clades: i.e. an Europe (E) and an Asia (A) clade with further Asian subclades (AS1, AS2 and THG) and a single not incorporated haplotype representing the Taiwanese Lanyu pig (see also Wu *et al.* 2007a). Twenty-three of the 24 Thai haplotypes arranged randomly in AS1 that is similar to the D2 cluster. This cluster is widely distributed among domestic Chinese and related pigs as well as the East Asian wild boars (Larson *et al.* 2005). Haplotype TNH19 represented only animals that derived from Mae Hongson province, haplotype TNH01 arranged to the European clade. Despite of the phenotypic differences, genetic exchanges of Thai pigs with exotic maternal lineages have occurred in the past, i.e by planned crossbreeding programs of the Thai government (Rattanaronchart 1994). Finally, eight of the 23 Thai haplotypes (i.e. 27 of the 78 tested individuals or a total of 34.6%) in clade AS1 recapitulated another major cluster (denoted Thai haplogroup = THG) that was supported by a high bootstrap value (Figure 3.1).

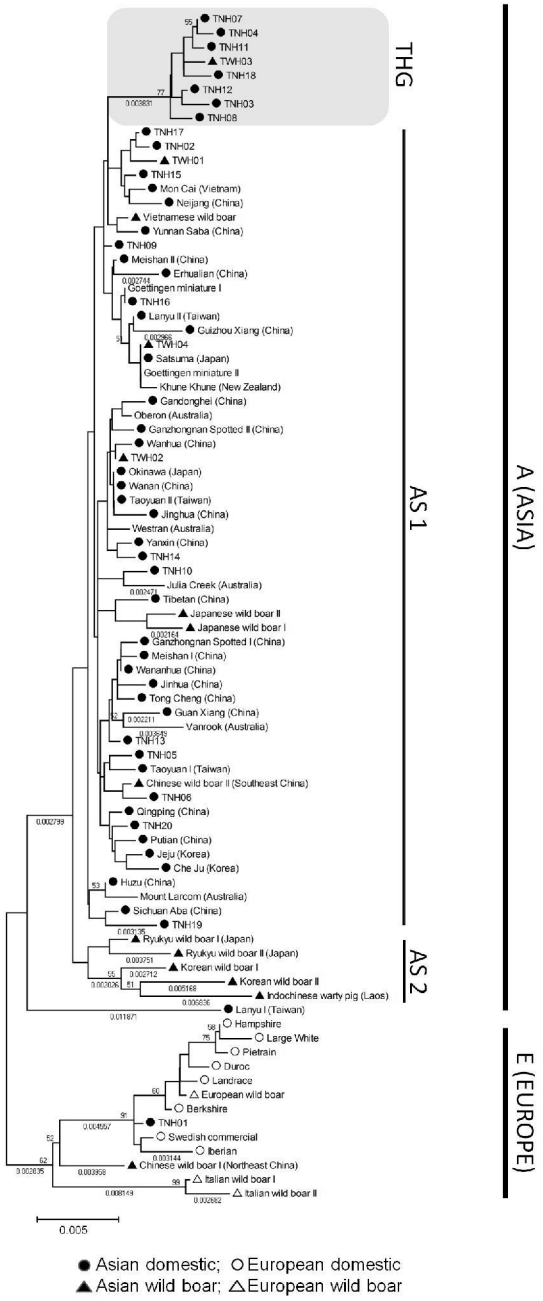

Figure 4.1 Neighbor-joining (NJ) phylogenetic tree of Thai, European and further Asian pigs. The tree consists of 84 mtDNA sequences and is presented in the bootstrap (10,000 replicates) consensus form. A total of 1044 bp of mtDNA was used. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown above the branches. The tree is drawn to scale with branch lengths (below the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Tanaka *et al.* (2008) have proposed that mtDNA haplotypes specific for pigs indigenous to the mountainous areas of Bhutan, Cambodia, Laos and Myanmar constitute a clade of their own (MTSEA). As the majority of these countries share borders with Thailand, we intended to investigate possible relationships of THG and MTSEA haplotypes using 510 bp of the mtDNA. A MJ (median joining) network according to Bandelt *et al.* (1995) was constructed using all variable characters of complete alignment and the software package NETWORK 4.5 (http://www.fluxus-engineering.com/ sharenet.htm). The star-like pattern network demonstrates that all MTSEA haplotypes – except haplotypes H29 (Bhutan pigs only) and H33 (Myanmar pigs only) – are integrated into the THG haplogroup or vice versa. The MTSEA-THG haplogroup is shown in Figure. 3.2.

Recently, MTSEA has been identified as a mixed clade 3 of wild and domestic samples (MC3) that were found almost exclusively in the Indo-Burma Biodiversity Hotspot (IBBH) that includes Laos, Cambodia, Myanmar and Thailand to the Kra Isthmus (Larson *et al.* 2010). The high degree of genetic diversity within the IBBH is most probably the consequence of demographic expansions of agricultural populations and thus domestic pigs into this area (Larson *et al.* 2010). Moreover and in particular, the MTSEA-THG haplogroup (Figure 3.2) also displays the history of Thailand, as it represents only haplotypes that are indigenous to provinces that previously belonged to the former Thai Kingdom (Siam). Genetic exchange between these provinces was always possible because of transport routes (i.e. the Mekong river) and migration of people between provinces. The Franco Siamese War (1867–1909) led finally to territorial losses of the Siamese Kingdom when Shan State (13) became part of Myanmar. In addition, provinces Vientiane (7), Xiang Khoang (8), Borikamxai (9) and Champasak (10) went to Laos and provinces Ratanakiri (11) and Moldukiri (12) to Cambodia (Baker & Phongpaochit 2005).

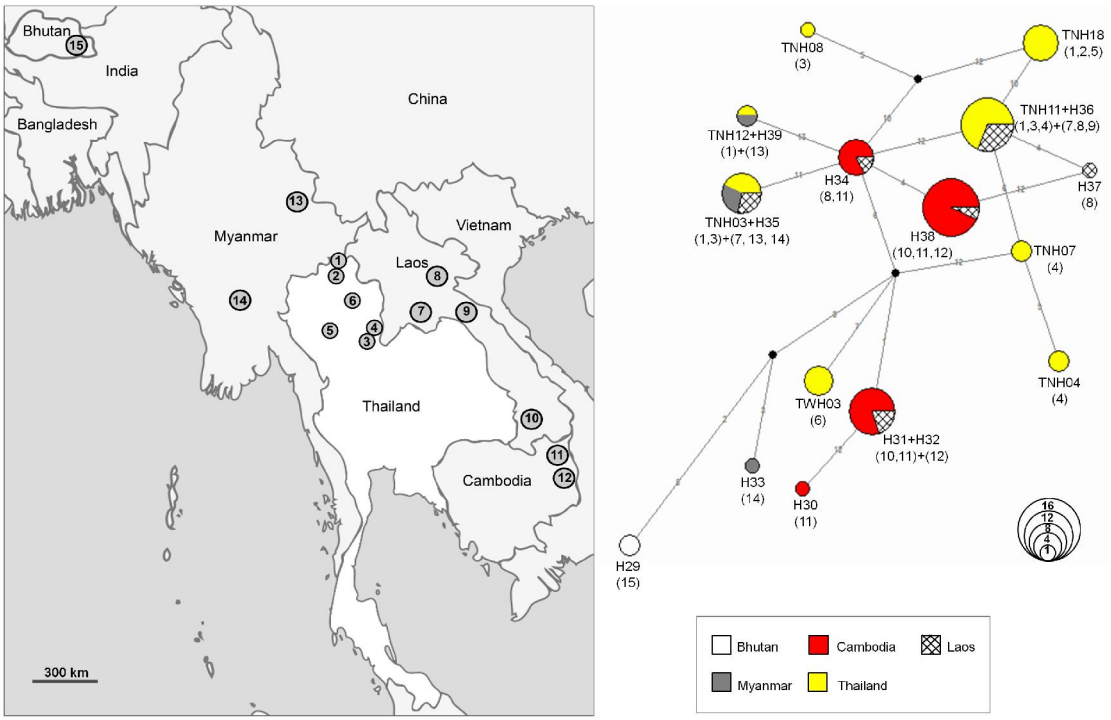

Figure 3.2 Reduced median-joining network of THG and MTSEA haplotypes including the location of haplotypes. Considered for the analysis were 510 bp of the mtDNA. The circle size corresponds to the haplotype frequency. If animals of one population possessed more than one haplotype, the pie illustrates the respective proportions. The Median vector (mv) algorithms calculate hypothetical sequences that are illustrated by small solid dots. The numbers in parenthesis refer to the sampling locations. Sampling locations in Thailand are (1) Chiang San, (2) Viang Chai, (3) Nam Pat, (4) Fak Tha, (5) Tung Huachang, and (6) Ban Luang. Sampling locations in Laos are (7) Vientiane, (8) Xiang Khoang, (9) Borikamxai, and (10) Champasak. Sampling locations in Cambodia are (11) Ratanakiri, and (12) Moldukiri. Sampling locations in Myanmar are (13) Shan State and (14) Bago division, the respective sampling location in Bhutan is (15) Mongar. Circles TNH11+H36, TNH03+H35 and TNH12+H39 combine haplotypes of this study and of Tanaka *et al.* (2008). The most geographically distant haplotype H29 (Bhutan pigs only) is separated by two mutation steps.

The assignment of the 15 porcine Thai haplotypes to cluster AS1, supports the hypothesis of a shared common ancestors with the Chinese domestic pigs, but the formation of the separate MTSEA-THG clade is also most putatively an indication for a further independent domestication event in Southeast Asia (SEA) in the past. All members of the MTSEA-THG haplogroup have also revealed unique signatures at

position 24 (nucleotide A) and at position 183 (nucleotide C) (according to positions 25 and 184 in Tanaka *et al.* 2008) that differentiate them from all other known porcine haplotypes. In addition, each Thai haplotype possesses a further unique signature (nucleotide G) at position 896. Its existence in MTSEA-THG haplotypes cannot be solved here. Tanaka *et al.* (2008) did not provide any data concerning this part of the mtDNA.

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Supplementary Materials

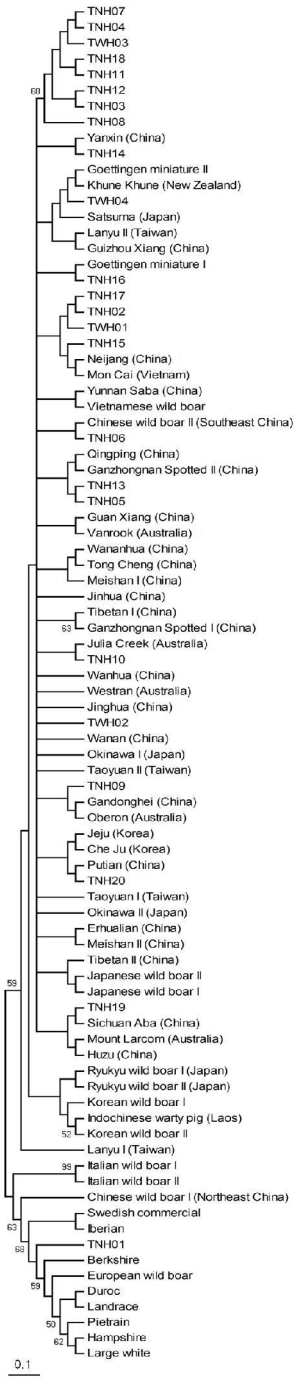

Figure S3.1 Maximum likelihood consensus tree of Thai, European and further Asian based on 84 mtDNA sequences.

CHAPTER 4

Microsatellite Analysis of Thai Native and Commercial Pigs

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Microsatellite analysis of Thai native and commercial pigs

Abstract

The present study aimed to genetically characterize Thai indigenous pigs collected from 12 locations of study area in five provinces of Northern Thailand by typing 26 microsatellite markers. The comparison of their genetic background with commercial pigs in Thailand and with some Chinese pigs were investigated. The results showed that Thai indigenous pig populations had a high genetic diversity with mean total (TNA) and effective (N_e) number of alleles of 14.59 and 3.71, respectively, and an expected heterozygosity (H_e) of 0.710 across loci. The polymorphic information content (PIC) per locus ranged between 0.651 and 0.914 leading to an average value above all loci of 0.789. The private microsatellite alleles were found here in six pig population with nine markers of the 26 marker analyzed, mostly in Thai indigenous pigs. Thai native pigs and Thai wild boars had advantageous H_e and H_o compared to the commercial pigs, but not always to the crossbreds that revealed a surplus of heterozygous animals. The calculated inbreeding coefficient was zero in the crossbreds and also low in all of the commercial breeds. The Nei' s genetic distance, mean F_{ST} estimates, neighbor-joining tree of populations and individual as well as multidimensional analysis indicates the close genetic relationship between Thai indigenous pigs and some Chinese pigs, and they are distinctly different from European pigs. However, the genetic introgression from European commercial breeds were found in some Thai native pigs. All of above genetic analyses showed that Thai native pig populations are unique genetic resources.

Keywords: genetic diversity, microsatellites, native pigs, Thailand.

Introduction

European pigs (e.g. Duroc, Large White, and Pietrain) have been imported to Thailand in the 50's of the last century (Visitpanich and Falvey 1980), but not until the 80's

they have been increasingly mated with indigenous pigs. Their genetics improved the performance of economically important traits and industrialized the Thai pork industry. Finally, they completely replaced the native pigs in the market so that the number of indigenous pigs in Thailand has steadily decreased over the years. Indigenous Thai pigs (native pigs and wild boars) are nowadays particularly kept by smallholders in the Northern and North Eastern provinces of Thailand for the sake of tradition and of religion (Nakai 2008; Rattanaronchart 1994). An already in 1994 performed survey (Rattanaronchart 1994) reported that less than 500 herdbook sows and less than 10 nucleus herds were registered. Rattanaronchart postulated therefore that indigenous Thai breeds are on the edge of extinction.

The conservation and also the utilization of indigenous genetics has become a big challenge in agricultural sciences: phenotypes (including the performance traits) as well as the genetic background have to be described and recorded to achieve this goal (Rege and Okeyo 2006). Microsatellite markers have proven to be an extremely powerful tool to analyze the genetic diversity and the phylogenetic relationships in pigs (e.g. Kim *et al.* 2005; Vicente *et al.* 2008; Fang *et al.* 2009). In contrast to other pig breeds/sources, only one study was conducted so far to investigate the genetic background of the Thai native pig breed (Chaiwatanasin *et al.* 2002; Charoensook *et al.* 2009).

The goal of our study was thus a large-scale analysis of indigenous breeds, crossbreds and commercial exotic breeds in Thailand to assess their genetic diversity. To study their phylogeny, we included also Chinese breeds.

Materials and Methods

Resource populations

Samples of 72 Thai native pigs and of 11 Thai wild boars were used. Blood, ear clips or hair samples were collected in twelve localities/amphurs in five Northern provinces, i.e. Mae Hongson, Chiang Mai, Chiang Rai, Uttaradit and Nan. The Thai native pigs were assigned to one of the five native pig populations (Table 4.1). Thai wild boars are assigned to one population, as they were originally caught in the wilderness of the provinces Chiang Mai und Nan. In addition three populations of purebred European pigs

and of two crossbreds between European and Thai pigs were investigated. These 11 populations are further called 'Thai pigs'. Finally, six Chinese pig breeds (taken from the DNA repository of the Institute of Veterinary Medicine, Göttingen, Germany) were used as a reference to compare the genetic diversity of Thai pigs with further Asian pig sources. The Chinese pigs were selected based on different geographical distributions and ecological types reflected by different phenotypic and morphological characters. The Chinese pigs were as follows: The lower Yangtze river basin type Jiangquhai (JQH), the South China type Luchuan (LC), the North China type Minpig (MZ), the Southwest type Rongchang (RC), the Central China type Yushanhei (YJ) and the plateau type Tibetan (TI) with 10 animals each (Chen *et al.* 2005). In total, 222 individuals were analyzed.

Table 4.1 Animals and sampling information

Local or breed sample	Sampling location	Abb.	N	Sampling type
Native pig I	Mae Hongson	MH	6	Ear clip
Native pig II	Southern part of Chiang Mai	SCM	29	Blood, ear clip, hair
Native pig III	Northern part of Chiang Mai	NCM	20	Blood, ear clip
Native pig IV	Chiang Rai	CR	11	Blood, ear clip
Native pig V	Uttaradit	UT	21	Blood, ear clip
Wild boar	Chiang Mai & Nan	TWB	11	Blood, ear clip
Duroc	Chiang Mai	DR	22	Blood
Pietrain	Chiang Mai	PT	10	Blood
Large White	Chiang Mai	LW	12	Blood
Duroc × native crossbreds	Chiang Mai	DXN	10	Blood
Pietrain × native crossbreds	Chiang Mai	PXN	10	Blood
Jiangquhai	Jiangsu Province	JQH	10	Blood
Luchuan	Guangxi Province	LC	10	Blood
Min	Liaoning, Jilin & Heilongjiang	MZ	10	Blood
Yushanhei	North-Eastern Jiangxi	YJ	10	Blood
Tibetan	Tibet & Yunan	TI	10	Blood
Rongshang	Western Sichuan	RC	10	Blood
Total			222	

Molecular genetics analyses

Genomic DNA was extracted from whole blood (9 ml vials containing EDTA) and ear clips by a modified salting out method according to Sambrook *et al.* (1989) and Miller *et al.* (1988) or from hair roots using the QIAamp DNA mini kit (Qiagen, Germany).

A panel of 26 microsatellites was analyzed that covered all porcine chromosomes including the sex chromosomes (Table 4.2 and Table S4.1). Primers were fluorescently labeled with dyes FAM or HEX at the 5'-end. PCR assays were performed using 50-100 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer and 0.5 units of *Taq* polymerase (Qiagen, Germany) in 1x PCR buffer as recommended by the manufacturer in a final volume of 25 ul. The PCR profile consisted of 35 cycles at 94 ºC for 30 sec, the specific annealing temperature for 30 sec (see Table S1), and an extension period of 30 sec at 72 ºC with an initial denaturation for 2 min at 94 ºC and a final extension at 72 ºC for 5 min. PCR reactions were performed on a Biometra T-Gradient thermocycler (Biometra, Germany). To check fragment integrity PCR products were loaded on 2% agarose gels.

Genotyping

For genotyping of samples, the size separation was performed on an ABI PRISM® 3100 DNA analyzer (ABI, Weiterstadt, Germany), using GENESCAN™-500ROX™ as internal size standard according to the manufacturer' s specifications. Evaluation of microsatellites and size determination of alleles were done with appropriate ABI-softwares GENESCAN and Gentoyper software (Applied Biosystems, Applera Europe B.V.), respectively.

Statistical analyses

Several softwares (Table S4.2) were applied to calculate parameters for each microsatellite, for each population or for each analyzed individual. GENETIX 4.03 (Belkhir *et al.* 1998). resp. POPGENE 1.31 (Yeh et al. 1999) were used to compute the observed heterozygosity (H_O), the expected heterozygosity (H_E), the mean number of alleles (MNA) and the effective number of allels (N_E) per microsatellite resp. population. CERVUS 3.0.3

(Marshall *et al.* 1998) was used to calculate the polymorphism information content (PIC) per locus (Botstein *et al.* 1980) and possible deviations from the Hardy-Weinberg equilibrium (HWE). FSTAT 2.9.3 (Goudet 2001) was applied to assess F-values. POPGENE 1.31 was also used to determine Nei genetic distances between populations. NJ-trees were obtained by PHYLIP (Felsenstein, 1993-2002) and by MEGA 4 (Tamura *et al.* 2007). Finally, GENEALEX (Peakall & Smouse 2006) computed the mean Fst distances and provided two dimensional data (MDS-2D) data to project objects based on their similarity or dissimilarity between each other.

Results and Discussion

Studies to document the genetic diversity of indigenous pig breeds and to decipher phylogenetic relationships with further breeds or lines using microsatellite markers are numerous (e.g. Zhang *et al.* 2003; Vincente *et al.* 2008; Megens *et al.* 2008; Fang *et al.* 2009). Despite that the ISAG/FAO Standing Committee for biodiversity has recommended a panel of 27 pre-selected microsatellites (ISAG/FAO, 2004) this has been used only in few times. We used 24 of the recommended 27 microsatellites. Three STS-markers S0178, S0228 and SW24 -presented unreliable standard- were replaced in our survey by markers S0120 and SW1031 to cover all porcine chromosomes including the sex chromosomes.

Microsatellite diversity

In total, 367 alleles were observed at the 26 loci. The total number of alleles per locus (TNA) varied from 7 (SW951) to 29 (CGA) with a global mean of 14.59 alleles per locus (Table 4.2). All microsatellites revealed high degrees of polymorphism and allelic diversity. MNA per marker ranged between 3.82 (SW951) and 10.64 (CGA) with an overall mean of 5.65. N_e ranged between 2.62 (S0218) and 7.15 (CGA) with a pooled mean of 3.71. For nine of the 26 loci private alleles were described. The highest number of specific alleles per marker was visible for SW122 (three). Only for three alleles, frequencies of 10% or higher were observed (allele 229 at S0227; allele 107 at SW122, and allele 255 at S0068). Genotyping of further individuals should help to verify at the

population level which of these alleles are at low frequency or not at all present in the respective pig sources. The highest frequency of specific alleles per population was observed for TWB and pigs collected in the Uttaradit province. Our data support Thuy et al., (2006) who also reported new alleles per locus present in the indigenous breeds in their comparative study of Vietnamese and European pigs.

The polymorphic information content (PIC) per locus was highest (0.914) for CGA and lowest (0.651) for SW951 leading to an average value above all loci of 0.789, which is superior to the one of 0.755 of the Thai pigs investigated by Chaiwatanasin *et al.* (2002) and the one of 0.685 reported for Portugese breeds (Vincente *et al.* 2008). The overall H_o for our Thai pigs was 0.679 and the H_e was 0.710. Vincente *et al.* (2008) reported lower values of 0.621 resp. 0.667 for H_o resp. H_e. For Mexican Creole pigs values of 0.46 ± 0.04 (H_o) and of 0.72 ± 0.04 for H_e were described (Lemus-Flores *et al.* 2001). Fabuel *et al.* (2004) introduced a H_o of 0.576 and a H_e of 0.697 calculated for their Iberian pigs. Finally, Chaiwatanasin *et al.* (2002) documented a mean observed heterozygosity of 0.534 and a mean expected heterozygosity of 0.793. Wright's *F*-statistic estimates were calculated for each locus (Table 4.2). The divergence between expected and observed heterozygosities for all individuals is documented by the total inbreeding estimate (F_{IT}), which amounted to 0.169 (variation between 0.013 for S0226 and 0.583 for S0386). This value is lower compared to those of 0.38 resp. 0.33 in Chinese pig breeds (Li *et al.* 2004 resp. Yang *et al.* 2003) and the one of 0.239 reported for Portugese pigs (Vincente *et al.* 2008).

We observed a within-population inbreeding coefficient F_{IS} of 0.007 that was lower than the one reported by others for their populations (Yang et al. (2003), F_{IS} = 0.274; Vincente *et al.* (2008), F_{IS} = 0.067; Li *et al.* (2004), F_{IS} = 0.21). Nevertheless, *S0386* and *S0218* had extremely high F_{IS} estimates (0.518 and 0.377) that were in accordance to published data for *S0386* but conflicting for the X-linked marker S0218 (Yang *et al.* 2003). F_{IS} reflects excess or reduction between H_o and H_e and might be caused by null alleles or population subdivisions (Maudet *et al.* 2002).

The multi-locus F_{ST} that mirrors the population differentiation averaged to 0.162. There were variations in the single-locus F_{ST} values from 0.072 (CGA) to 0.249 (S0218). This is an indication that not more than about 16% of the total genetic variation can be explained by differences between the investigated populations. The

majority of the genetic diversity is thus an effect within populations. A comparison with the data provided by Yang *et al.* (2003), Li *et al.* (2004) and Vincente *et al.* (2008) demonstrate that the values were in the range of these studies (0.077 to 0.022).

A comparison with literature data is, however, somehow biased as the number of observations varied and different marker sets with an uneven number of markers were used. Despite that Thai pigs seem to be genetically more diverse and less inbred than the further indigenous pig breeds. To finally support or reject this hypothesis, a comparison test using a joint platform to avoid allele mis-calling should be conducted.

Genetic diversity in Thai pig populations

Table 3 depicts the assessment of the genetic diversity in the Thai pig populations. Over all, MNA and N_e were elevated in TNP and TWB. Only MH revealed less superior values: a lower MNA and a lower N_e compared to some of the commercial pigs and crossbreds. The deviations were, however, not significant. An elevated allelic diversity in indigenous breeds is known (e.g. Thuy *et al.* 2006) and probably the consequence of the lack of planned mating programmes. Thai native pigs and Thai wild boars had advantageous H_e and H_o compared to the commercial pigs, but not always to the crossbreds that revealed a surplus of heterozygous animals ($H_o > H_e$). A high heterozygosity must be attributed to heterosis, and at the same time to a marginal degree of inbreeding effects. In their earlier study, Chaiwatanasin *et al.* (2002) described estimates for H_e and H_o in Thai native pigs (TN) and commercial Thai pigs represented by the breeds Large White (LW), Pietrain (PT), and Spotted Large White (SLW). The authors reported only small differences of the H_O between the populations. Unexpectedly, they also documented a higher H_o in LW and SLW – but not for PT - compared to the TN animals. The highest H_E was computed for the TN. Chaiwatanasin's TN pigs revealed a broader H_e to H_o ratio (0.534 and 0.793) compared to our pigs as we reported higher H_o. Unfortunately, Chaiwatanasin *et al.* (2002) did not provide sufficient information about the origin of the animals to interpret the data and to deduce more detailed possible reasons for the deviations.

Table 4.2 Characterization of the 26 microsatellites in Thai pigs[1]

Locus	SSC	TNA	MNA	N_e	PIC	H_o	H_e	F_{IT}	F_{ST}	F_{IS}	HWE	Private allele; bp (animal & frequency)
S0155 *	1	10	5.73	3.58	0.835	0.736	0.733	0.139	0.179	-0.049	NS	143 (UT 0.05)
CGA *	1	29	10.64	7.15	0.914	0.761	0.889	0.171	0.072	0.107	**	
S0226 *	2	11	4.45	3.04	0.761	0.785	0.669	0.013	0.193	-0.224	*	
SW240 *	2	14	5.36	3.60	0.828	0.741	0.708	0.127	0.199	-0.091	**	120 (UT 0.07)
S0002 *	3	27	7.91	5.25	0.896	0.839	0.794	0.068	0.155	-0.103	NS	204 (UT 0.05); 252 (NCM 0.05)
SW72 *	3	10	5.36	2.95	0.697	0.617	0.656	0.141	0.124	0.020	NS	
S0227 *	4	9	4.09	2.85	0.707	0.683	0.648	0.068	0.154	-0.102	NS	229 (TWB 0.14)
IGF-1 *	5	10	5.36	3.84	0.795	0.765	0.738	0.060	0.131	-0.082	NS	
S0005 *	5	19	7.00	4.73	0.861	0.671	0.810	0.241	0.123	0.134	ND	
SW122 *	6	13	5.64	3.49	0.845	0.728	0.712	0.156	0.208	-0.066	NS	99 (TWB 0.09); 107 (TWB 0.23); 109 (TWB 0.09)
S0101 *	7	11	4.73	2.89	0.778	0.633	0.654	0.234	0.241	-0.009	**	
SW632 *	7	11	6.00	3.58	0.823	0.635	0.732	0.248	0.168	0.095	**	
S0225 *	8	13	5.09	3.34	0.767	0.778	0.700	0.017	0.152	-0.160	NS	173 (NCM 0.05)
SW911 *	9	10	6.00	3.87	0.818	0.734	0.729	0.112	0.152	-0.047	*	144 (CR 0.09)
SW951 *	10	7	3.82	2.69	0.651	0.554	0.645	0.210	0.119	0.103	*	
S0386 *	11	9	4.18	2.84	0.663	0.293	0.635	0.583	0.135	0.518	**	
S0090 *	12	10	5.18	3.48	0.814	0.785	0.701	0.062	0.196	-0.167	NS	
S0068 *	13	17	7.45	5.08	0.884	0.878	0.802	0.018	0.141	-0.142	NS	255 (SCM 0.16)
S0215 *	13	13	4.45	3.07	0.737	0.553	0.580	0.251	0.246	0.007	**	
SW857 *	14	11	5.73	4.11	0.852	0.633	0.755	0.269	0.163	0.127	NS	
S0355 *	15	12	5.82	3.80	0.815	0.685	0.697	0.179	0.199	-0.025	NS	
SW936 *	15	18	6.09	4.12	0.836	0.840	0.764	0.029	0.153	-0.146	**	
S0026 *	16	9	5.09	3.35	0.762	0.731	0.715	0.083	0.140	-0.067	NS	
SW1031	17	11	4.82	2.99	0.696	0.498	0.659	0.319	0.137	0.211	**	116 (DR 0.07)
S0120	18	15	7.00	4.34	0.797	0.773	0.784	0.062	0.089	-0.029	NS	
S0218 *	X	11	4.09	2.62	0.685	0.335	0.562	0.532	0.249	0.377	**	
Mean		14.59	*5.65*	*3.71*	*0.789*	*0.679*	*0.710*	*0.169*	*0.162*	*0.007*		

[1]SSC = *Sus scrofa* Chromosome, OFS = Observed fragment size (bp), TNA = total number of alleles, MNA = mean number of alleles per population, N_e = effective number of alleles per population, H_o and H_e = observed and expected heterozygosity, PIC = polymorphism information content, F_{IT} = Total inbreeding estimate, F_{ST} = Estimate of population differentiation, F_{IS} = Within-population inbreeding coefficient, HWE = loci deviating from Hardy-Weinberg equilibrium test (* $P < 0.05$, ** $P < 0.01$, NS = non significantly different, ND = not done), Private alleles (analyzed with Thai and Chinese pigs).
*ISAG/ FAO, 2004.

The calculated inbreeding coefficient (*F*) was zero in the crossbreds and also low in all of the commercial breeds (Table 4.3). Heterosis is the main reason for the excess of heterozygosity in the crossbreds. The sophisticated breeding programs in commercial pigs should have led to their negligible degree of inbreeding. Unexpectedly high was instead the inbreeding in SCM (0.139), CR (0.105) and the TWB (0.100). Assuming no previous inbreeding between any parents, a *F* of at least 0.125 is expected if either grandfather/granddaughter (grandmother/grandson), alternatively half-brother/half-sister or uncle/niece (aunt/nephew) mating occurred. We cannot rule that out as animals of these populations were kept in small villages for generations (SCM and CR) or were caught and kept in captivity as in the case for the TWB.

Table 4.3 Genetic diversity of local or breed populations[1]

Local or breed population	MNA ± SD	N_e ± SD	H_o ± SD	H_e ± SD	*F*
Thai native pigs					
MH	4.46 ± 1.33	3.34 ± 1.32	0.721 ± 0.250	0.724 ± 0.119	0
SCM	8.23 ± 2.77	4.50 ± 1.71	0.638 ± 0.181	0.754 ± 0.107	0.139
NCM	6.15 ± 1.71	3.90 ± 1.26	0.726 ± 0.225	0.731 ± 0.115	0
CR	6.15 ± 2.03	3.99 ± 1.49	0.638 ± 0.229	0.746 ± 0.122	0.105
UT	7.84 ± 2.49	5.04 ± 2.20	0.721 ± 0.164	0.792 ± 0.079	0.066
Thai wild boar					
TWB	6.15 ± 1.28	4.28 ± 1.29	0.671 ± 0.214	0.782 ± 0.081	0.100
Commercial pigs					
DR	5.53 ± 2.46	3.23 ± 1.59	0.627 ± 0.217	0.641 ± 0.175	0
PT	4.50 ± 1.83	3.03 ± 1.41	0.574 ± 0.225	0.630 ± 0.186	0.017
LW	4.26 ± 1.11	2.84 ± 0.98	0.589 ± 0.181	0.632 ± 0.170	0.047
Commercial crossbred pigs					
DXN	4.07 ± 1.87	3.24 ± 1.35	0.853 ± 0.256	0.685 ± 0.121	0
PXN	4.96 ± 1.79	3.46 ± 1.12	0.712 ± 0.240	0.710 ± 0.131	0

[1]MNA = mean number of alleles per locus, N_e = effective number of alleles per locus, H_o and H_e = the observed and unbiased expected heterozygosity, *F* = heterozygote deficiency or inbreeding coefficient.

Genetic distances and phylogenetic relationships between pig populations

Genetic distances for the Thai and Chinese pig populations JQH, LC, MZ, YJ, TI, and RC were assessed according to Nei (Nei 1972) and by mean F_{ST} estimates (significance was

tested using the permutation test). Pair-wise comparisons for all pig populations are shown in Table 4. Nei's estimates indicated a higher than expected genetic distance between TWB and the further TNPs (from 0.352 to 0.606). Remarkable was indeed that the genetic distance between TWB and DR and PT (0.579 and 0.567) was even lower than the one to MH (0.606). Not unexpected, however, were the estimates for the crossbreds that lay somewhere in between the 'founder' breeds. Large genetic distances (≥ 0.439, expect for TI to RC) were also found between the Chinese breeds. There was e.g. one of 0.741 between LC and JQH. High genetic distances existed also between Thai and Chinese pigs. The closest relationships were, however, estimated between the Chinese breeds TI and RC and the Thai populations SCM and UT. The closest relationship of TWB with a Chinese breed was the one with TI. In fact, both populations are the least domesticated ones in their home-countries and graze in the forests instead of being housed in pens.

The overall F_{ST} of 0.162 indicates significant population subdivision over the Thai pig breeds. Laval *et al.* (2000) reported higher estimates for European breeds (F_{ST} = 0.27). Lower values are known for Chinese breeds (F_{ST} = 0.077; Yang et al., 2003). Our pair-wise F_{ST} estimates ranged from 0.037 (between CR and SCM) to 0.235 (between LW and LC). The F_{ST} estimated here were in the range of values reported by several authors for microsatellite analyses in pigs, with F_{ST} ranging from 0.11 to 0.27 in European breeds (Laval *et al.* 2000; Martinez *et al.* 2000; Vicente *et al.* 2008) and from 0.18 to 0.26 in Chinese and Korean breeds (Fan *et al.* 2002; Li *et al.* 2004; Kim *et al.* 2005). Nevertheless, these differences are most likely due to distinct sets of markers and the respective resource populations that are in any case a random sampling of a population (Kim *et al.* 2005). Finally, the broad tendency of the recorded F_{ST} values between TNP and European pigs is the same as reported by Chaiwatanasin *et al.* (2002). Contrary to that TWB are genetically more distant to European pigs.

A phylogenetic tree consisting of the 17 populations was reconstructed based on Nei's genetic distances (Figure 4.1) and distinguished two distinct clusters. The first cluster consisted of the three commercial breeds, the crossbreds, two TNP (NCM, MH) and two Chinese populations (MZ, JQH). The additional Chinese breeds arranged with the TWB and the Thai CR, SCM and UT and formed the second cluster.

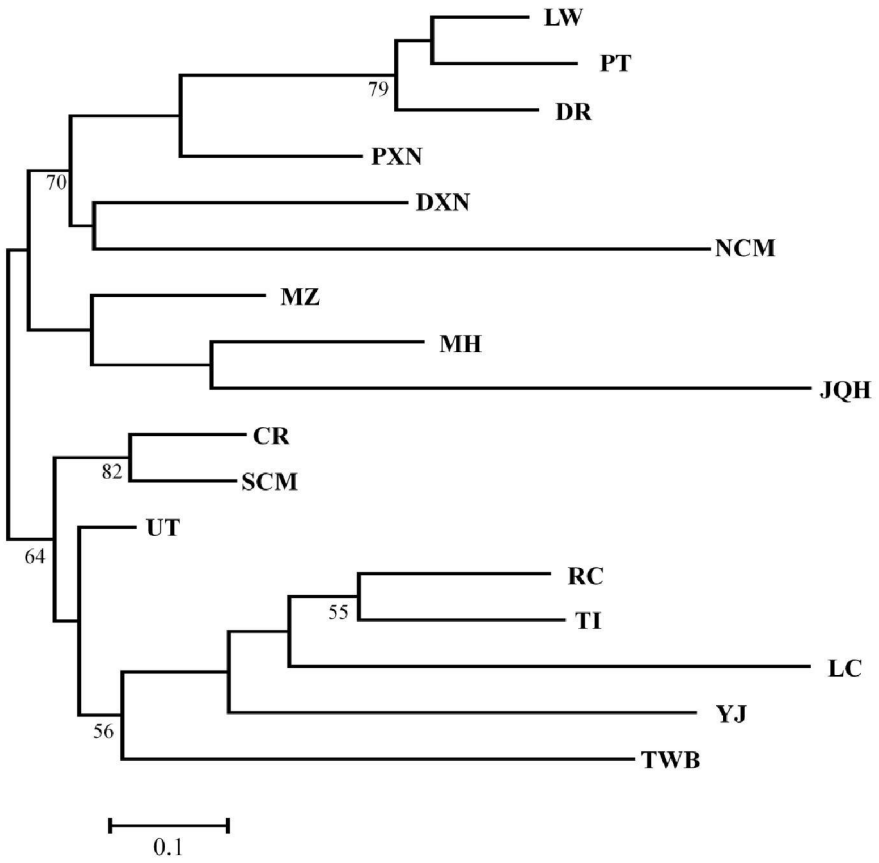

Figure 4.1 Representation of neighbor-joining Nei' s (1972) standard genetic distance among Thai and Chinese pig populations, based on 1,000 replicates (numbers in nodes are percentage bootstrap values). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are not shown.

Multidimensional scaling (MDS) was further computed to display genetic similarities among populations - based on the pair-wise proportion of different alleles (F_{ST}) - in a two-dimensional room (Figure 4.2). The European pigs separated clearly from each other, the crossbreds and the NCM populations formed a second group that separated the Asian breeds from the European breeds. This is a profound indication that crossbreeding events with individuals of commercial occurred in the NCM population.

Table 4.4 The Nei' s (1972) standard genetic distance (below the diagonal) and mean F_{ST} estimates (above the diagonal) among each pair of 17 populations

Pop.*	MH	SCM	NCM	CR	UT	TWB	DR	LW	PT	DXN	PXN	JQH	LC	MZ	YJ	TI	RC
MH	—	0.075	0.091	0.089	0.079	0.126	0.147	0.158	0.147	0.108	0.122	0.137	0.179	0.156	0.142	0.101	0.113
SCM	0.287	—	0.054	0.037	0.039	0.081	0.120	0.130	0.118	0.090	0.080	0.142	0.111	0.122	0.108	0.065	0.075
NCM	0.354	0.261	—	0.068	0.055	0.101	0.091	0.122	0.097	0.086	0.084	0.127	0.155	0.126	0.129	0.092	0.098
CR	0.312	0.127	0.300	—	0.046	0.085	0.107	0.118	0.103	0.095	0.086	0.160	0.129	0.127	0.122	0.086	0.093
UT	0.328	0.177	0.277	0.184	—	0.063	0.088	0.103	0.086	0.074	0.069	0.128	0.114	0.107	0.093	0.062	0.075
TWB	0.606	0.454	0.554	0.423	0.352	—	0.127	0.150	0.126	0.113	0.107	0.166	0.138	0.143	0.132	0.082	0.102
DR	0.558	0.551	0.354	0.418	0.371	0.579	—	0.082	0.064	0.095	0.111	0.199	0.199	0.168	0.183	0.131	0.149
LW	0.535	0.520	0.433	0.399	0.380	0.619	0.233	—	0.067	0.139	0.112	0.221	0.235	0.178	0.190	0.152	0.165
PT	0.544	0.526	0.377	0.388	0.356	0.567	0.185	0.160	—	0.097	0.113	0.208	0.209	0.149	0.181	0.130	0.146
DXN	0.370	0.406	0.348	0.383	0.319	0.526	0.317	0.448	0.318	—	0.114	0.155	0.182	0.145	0.153	0.114	0.130
PXN	0.474	0.369	0.358	0.353	0.305	0.520	0.407	0.335	0.408	0.450	—	0.182	0.166	0.138	0.148	0.103	0.118
JQH	0.459	0.588	0.474	0.629	0.532	0.702	0.712	0.719	0.735	0.534	0.686	—	0.222	0.190	0.187	0.138	0.147
LC	0.615	0.420	0.619	0.468	0.452	0.542	0.702	0.771	0.742	0.663	0.604	0.741	—	0.216	0.171	0.114	0.134
MZ	0.590	0.544	0.526	0.518	0.486	0.659	0.627	0.595	0.533	0.540	0.521	0.651	0.764	—	0.158	0.125	0.146
YJ	0.523	0.471	0.559	0.492	0.416	0.611	0.702	0.657	0.691	0.592	0.607	0.659	0.584	0.566	—	0.101	0.111
TI	0.462	0.350	0.524	0.456	0.369	0.484	0.646	0.665	0.628	0.567	0.519	0.579	0.431	0.582	0.442	—	0.055
RC	0.456	0.359	0.480	0.421	0.392	0.543	0.648	0.643	0.631	0.581	0.534	0.557	0.489	0.618	0.435	0.216	—

* Pop. = Population.

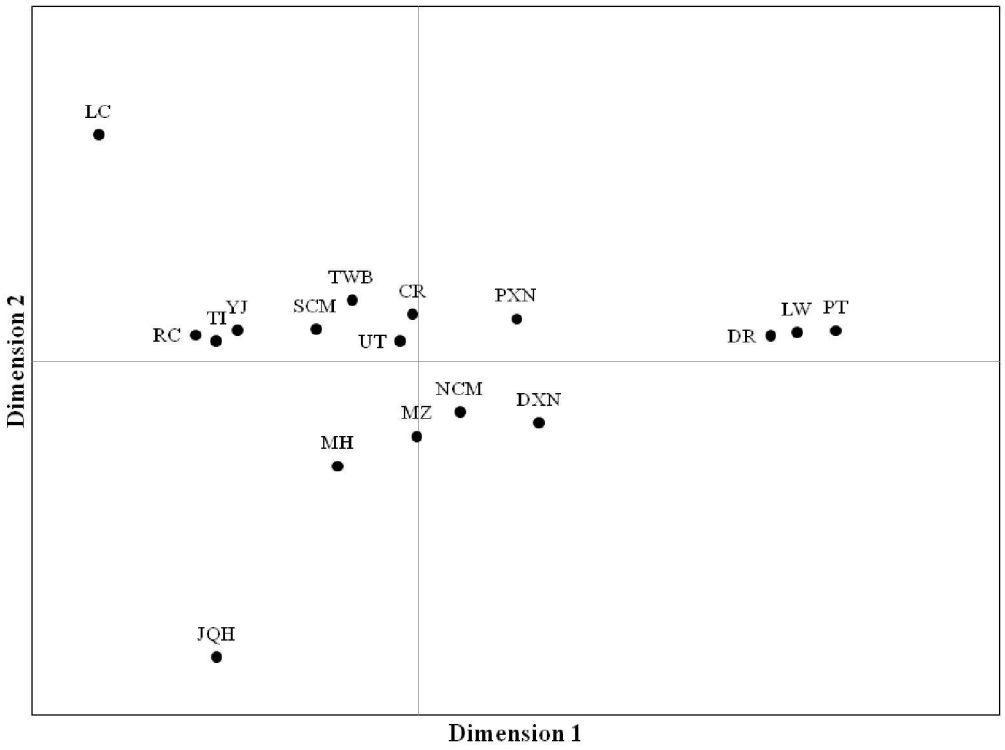

Figure 4.2 Multidimensional scaling (MDS) in a two dimensional area based on pairwise proportion of different alleles (F_{ST}) among 17 pig populations. Axis1 = 43.21 %, Axis2 = 25.22%.

The NJ tree of individuals was constructed based on Nei' s unbiased genetic distance (Nei 1978) of the proportion of shared alleles. The genetic structure and admixture of each pig populations (Figure 4.3). The phylogenetic tree proposed a close relationship between Thai native and Chinese pigs. At the same time both populations are distinctly different from European lineages. The tree did not reveal any geographic distribution of the breeds. In other words, we did not find a correlation between the geographic distance and genetic similarity. Any genetic differentiation among different breeds or populations was probably due to selection, drift, and local inbreeding effects. On the other hand, the close genetic relationship between the NCM and the crossbreds could be a direct effect of a genetic introgression from European pigs. In 1957, the Department of Livestock Development (DLD) at the Ministry of Agriculture, under the guidance of FAO, has started to import European pig breeds into Thailand and has promoted to raise and sale them in local areas. Thus, native pigs have been increasingly mated with European breeds (Rattanaronchart 1994).

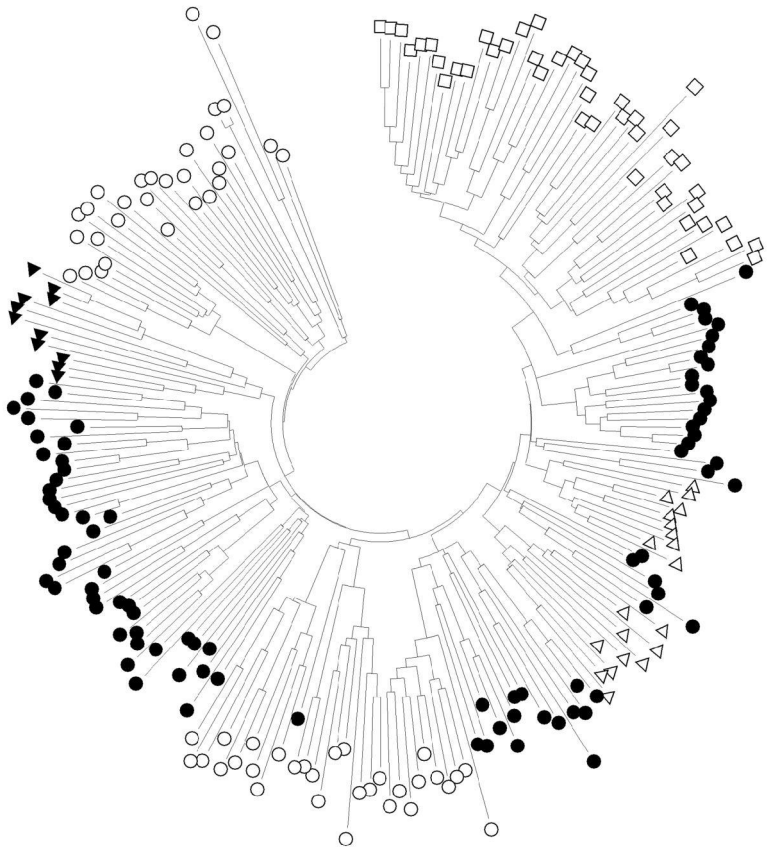

Figure 4.3 Neighbor-joining tree based on the proportion of shared alleles of Thai native pigs (black circle), wild boars (black triangle), Commercial crossbred pigs (white triangle), Commercial pigs (white square),and Chinese pigs (white circle).

What are possible implications of a porcine Thai genetic resource?

Thai native pigs are well adapted to hot and humid climates. They are probably better resistant to internal parasites and viral diseases (Rattanaronchart 1994). Kimloon (1998) described for example a low antibody titer (less than 40%) of 24 Thai native pigs in the province Lamphun against the food and mouth virus. All of pigs remained healthy with complete protection and no symptoms while the virus attached all cattle of the village. Hill tribe communities that predominantly raise Thai native pigs will presumably be the backups for a viable genetic resource of Thai native pigs. However, small pig populations without any scrutinized breeding programs are always at risk of losing genetic diversity. Recently, Charoensook *et al.* (2011) described specific mtDNA

signatures for our TNPs and TWB. The private microsatellite alleles that were reported here for UT (S0155, SW240, S0002), NCM (S0002, S0225), TWB (S0227, SW122), CR (SW911), SCM (S0068), and DR (SW1031) will provide additional information to genetically describe the uniqueness of Thai native pigs and Thai wild boars.

The present study have revealed the close genetic relationship between them and some Chinese pigs as well as the genetic introgression from European breeds. Some of local pig populations show sign of genetic erosion, clearly indicating that urgent measures of conservation and sustainable management of their gene pool must be undertaken. However, our conclusions should be considered in relation to the limited number of observations for some populations. The primary focus of the study was to evaluate the genetic of pigs in Northern Thailand that are the main genetic resource of native pigs in this country. Base on this study, however, we intend to discover with the large scale in the other part of Thailand. The provided genetic information is therefore a benefit for both conservation purposes as well as the utilization of them as an important genetic resource to improve future pig production in Thailand.

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Supplementary Materials

Table S4.1 Porcine microsatellite markers with chromosome location (SSC), annealing temperature (TA), expected fragment size (EFS), observed fragment size (OFS)

Marker	SSC	TA	EFS (bp)	OFS (bp)
S0155 *	1	53	150-166	141-165
CGA *	1	57	266-302	214-320
S0226 *	2	57	181-205	175-216
SW240 *	2	57	90-115	88-120
S0002 *	3	61	190-216	184-254
SW72 *	3	61	90-120	94-112
S0227 *	4	61	231-256	225-265
IGF-1 *	5	56	197-209	189-207
S0005 *	5	57	205-248	201-247
SW122 *	6	57	110-122	99-133
S0101 *	7	56	197-216	195-223
SW632 *	7	57	159-180	144-176
S0225 *	8	52	170-196	167-193
SW911 *	9	61	153-177	144-172
SW951 *	10	59	120-136	120-132
S0386 *	11	50	156-172	150-190
S0090 *	12	58	244-251	229-249
S0068 *	13	64	211-260	207-257
S0215 *	13	64	135-169	123-183
SW857 *	14	61	144-160	138-164
S0355 *	15	56	243-277	241-269
SW936 *	15	57	80-117	75-119
S0026 *	16	59	92-106	85-111
SW1031	17	57	93-107	87-116
S0120	18	57	154-176	148-181
S0218 *	X	61	166-204	156-188

* ISAG/ FAO (2004)

Table S4.2. Applications and software for microsatellite analysis

Applications	Software
MNA, Ne, Ho, He (each microsatellite)	Genetix 4.03 (Belkhir *et al.* 1998)
PIC, HWE test	CERVUS 3.0.3 (Marshall *et al.* 1998)
F-value	FSTAT 2.9.3 (Goudet 2001)
MNA, Ne, Ho, He (each population)	POPGENE 1.31 (Yeh *et al.* 1999)
Nei genetic distance (population)	POPGENE 1.31 (Yeh *et al.* 1999)
Mean Fst distance	GENEALEX (Peakall & Smouse 2006)
MDS-2D	GENEALEX (Peakall & Smouse 2006)
NJ-tree (population)	PHYLIP (Felsenstein, 1993-2002); Tree view 1.6.6 (Page 2001)
NJ-tree (individual)	MEGA 4 (Tamura *et al.* 2007)

CHAPTER 5

Polymorphisms in the Bovine *HSP90AB1* Gene are associated with Better Heat Tolerance in Thai Indigenous Cattle

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Polymorphisms in the bovine HSP90AB1 gene are associated with better heat tolerance in Thai indigenous cattle

Abstract

Heat shock proteins act as molecular chaperones that have preferentially been transcribed in response to severe perturbations of the cellular homeostasis such as heat stress. Here the traits respiration rate (RR), rectal temperature (RT), pack cell volume (PCV), and the individual heat tolerance coefficient (HTC) were recorded as physiological responses on heat stress (environmental temperatures) in *Bos taurus* (crossbred Holstein Friesian; HF) and *Bos indicus* (Thai native cattle: White Lamphun; WL and Mountain cattle; MT) animals (n = 47) in Thailand. Polymorphisms of the heat shock protein 90-kDa beta gene (*HSP90AB1*) were evaluated by comparative sequencing. Nine SNPs were identified, i.e. three in exons 10 and 11, five in introns 8, 9, 10, 11, and one was located in the 3'UTR. The exon 11 SNP g.5082 C>T led to a missense mutation (alanine to valine). During the period of extreme heat (in the afternoon) RR and RT were elevated in each of the three breeds, whereas the PCV decreased. MT and WL were superior in all physiological traits compared to HF indicating breed specific physiological responses to heat stress. The association analysis revealed that the T allele at SNP g.4338T>C within intron 3 improved the heat tolerance ($p < 0.05$). Allele T was exclusively found in WL animals and to 84% in MT. HF cattle revealed an allele frequency of only 18%. Polymorphisms within *HSP90AB1* were not causative for the physiological responses, however, we propose that they should at least be used as genetic markers to select appropriate breeds for hot climates.

Keywords: Heat stress, *HSP90AB1*, Polymorphisms, indigenous cattle, Thailand

Introduction

Global warming and the proposed climate change are likely to become the major threats to the sustainability of livestock production systems in the future. Simulations of

different climate scenarios suggest regional increases in temperature. In addition the intensity and duration of heat waves will dramatically go up (Gaughan *et al.* 2010). The changes will cause pertinent heat stress to livestock visible as predicted by e.g. a reduced feed consumption rate (Bernabucci *et al.* 1999), a decreased milk production (Sharma *et al.* 1988) and a lower reproductive success rate (Cavestany *et al.* 1985).

The physiological mechanisms of heat stress regulation are known to be identical in *Bos taurus* and *Bos indicus* cattle. *Bos indicus* is, however, generally better adapted to heat stress (Beatty *et al.* 2006): several studies report that the detrimental effects of heat stress on production traits are of a lesser extent (Gaughan *et al.* 1999; Gaughan *et al.* 2010). Mammals respond to heat stress with an evolutionary old and conserved adaptive cellular system. It is characterized by the transcriptional activation and accumulation of a set of proteins known as heat shock proteins (HSP). Isoforms of these proteins are categorized into families with respect to their molecular weight i.e. HSP27, HSP60, HSP70, HSP90 and HSP110/104 (Kregel 2002).

90-kDa heat shock proteins (Hsp90) act as important molecular charperones that are constitutively expressed as a consequence of heat or stress induction (Chen *et al.* 2006). Two major cytoplasmatic Hsp90 isoforms constituted by gene duplication: the inducible Hsp90α and the constitutive Hsp90β form. The contribution of Hsp90 isoforms to various cellular processes including signal transduction, protein folding, protein degradation, cell survival, and morphological evolution has extensively been studied (Csermely *et al.* 1998). A quantitative trait locus (QTL) study in *Drosophila melanogaster* (Morgan & Mackay 2006) mapped heat stress resistance to a genomic region on chromosome 3 containing amongst other genes the positional candidate gene including *HSP83*, which is the ortholog to the mammalian *HSP90* gene family (Marcos-Carcavilla *et al.* 2010). In sheep polymorphisms within another Hsp90 gene - the *HSPAA1* - were investigated. SNP located at position -660 in the 5'flanking region was associated with different thermal conditions (Marcos-Carcavilla *et al.* 2010).

The objective of this study was to record physiological parameters along with heat stress, to search for sequence variants in *HSP90AB1* and to describe putative associations between them in three cattle breeds used in Northern Thailand.

Materials and Methods

Experimental animals

Fourty-seven clinically healthy not lactating females between 12 and 18 months were randomly selected and kept at the experimental farm of the Chiang Mai University in Thailand. The animals belonged to the indigenous *Bos indicus* breeds White Lamphun (WL; n = 17) and Mountain cattle (MT; n = 16) as well as to a crossbred of *Bos indicus* and *Bos taurus* animals - further called Holstein Friesian (HF; n = 14) - with a proportion between 82.8% and 98.4% Holstein Friesian blood. The indigenous Thai cattle were kept in groups according to the animal welfare rules at the experimental farm under natural conditions. They were fed ad libitum on seasonal grass, rice straw and fresh water.

Physiological parameters

Respiratory rate (diaphragm movements per minute) (RR) and rectal temperature (RT) (ºC) were measured in the morning (8.00 am) and in the afternoon (2.00 pm). In addition blood samples were collected according to the recommendation of the manufacturer in capillaries to measure the pack cell volume (PCV) - percentage of red blood cells in the plasma - using a hematocrit centrifuge (HAEMATOKRIT-210; Hettich, Germany). The measurements and the sample collection were performed two weeks per month for four consecutive months (September to December) to achieve 8 observations per animal. The outdoor temperature and the relative humidity (RH) (%) were recorded daily during the experiment.

Earlier heat tolerance experiments led to the development of a formula (Rhoad 1944) to calculate an individual's heat tolerance coefficient (HTC). This formula - also known as the Iberia heat tolerance test for cattle - is as follows:

$$HTC = 100 - 10\,(ART - 38.3),$$

where HTC is the heat tolerance coefficient; ART is the average rectum temperature; 38.3 is the physiological bovine body temperature; 10 is a correction factor to convert deviations in body temperature to a unit basis, and 100 is the perfect efficiency in

maintaining temperature at 38.3 ºC. The index of HTC was calculated for each cow to assess its heat adaptability.

Molecular genetics analyses

Genomic DNA was extracted for all experimental animals from whole blood (9 ml vials containing EDTA) and/or from ear clips with a modified salting out method according to (Sambrook *et al.* 1989) and (Miller *et al.* 1988). Twelve DNA samples (four Holstein Friesian crossbreds, four White Lamphum and four Mountain cattle) of Thai cattle and twelve samples (four German Holstein Friesians, four Holstein Reds and four Charolais) of the DNA repository at the Institute of Veterinary Medicine in Göttingen (Germany) were randomly chosen to screen for Single Nucleotide Polymorphism (SNP) by comparative sequencing.

Six primer combinations were created based on the publicly available bovine *HSP90AB1* gene sequence (Acc. No. NW001494158). The PCR products cover exons 2 to 12 (Table 1). PCR was carried out using 50 to 100 ng of genomic DNA, 0.2 mM dNTPs, 40 pM of each primer and 2.5 U of Taq DNA polymerase in 1x PCR buffer in a final volume of 25 µl. The PCR profile used was: 35 cycles at 94 ºC for 30 sec, an primer specific annealing temperature (see Table 5.1) for 30 sec, and an extension period of 30 sec at 72 ºC with an initial denaturation for 2 min at 94 ºC and a final extension at 72 ºC for 5 min. PCR reactions were performed using the Biometra T-Gradient thermocycler (Biometra, Germany). To check fragment integrity PCR products were separated on 1% agarose gels. PCR products were then purified with the QIAquick PCR Purification Kit (Qiagen, Germany). The purified PCR products were directly sequenced using the BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit® (Applied Biosystems, Germany) on an automated DNA sequencer (ABI-PRISM 3100® capillary analyzer; Applied Biosystems, Germany). The sequenced data were analyzed and manually checked using the software suite DNASTAR LasergeneTM 6® (DNASTAR, Inc., Germany).

Table 5.1 Primers for PCR screening of *HSP90AB1* gene

Primer	Primer sequences (5' → 3')	Position[1]	Size (bp)	Tm (ºC)
Ex2Af	CCTGGATTGGAATGCCTAAC	1160	734	61.6
Ex3Br	TCAGGCTCTCATAGCGAATC	1894		
Ex3Af	AGGGAGTAATCAGAATAAG	1777	934	58.7
Ex5Ar	AGATGACAGTTTCAGAGTG	2711		
Ex6Af	TCACCCAGGAGGAATATGGAG	2981	692	61.6
Ex8Br	AGAAGGACCGATTTTCTCACC	3673		
Ex8Af	TTAAGGATCCTCTGCAGCAC	3638	710	61.6
Ex10Br	GCAACCTGCTCTTTGCTCTC	4348		
Ex9Af	TCTATTACATCACTGGTGCG	4207	654	61.6
Ex10Cr	TGTTGGAGATCGTCACCTG	4861		
Ex10Af	AGGTGGAGAAGGTAAGCCATT	4604	1040	62.9
Ex12Br	GTGTAAAAAACCAGCATCTTC	5664		

[1]Numbers refer to GenBank Acc. No. NW001494158

Statistical analyses

Gene diversity, allele and genotype frequencies and their accordance with or deviation from the Hardy-Weinberg law were determined by POPGENE 1.31 (Yeh *et al.* 1999) and GenAlEx 6.3 (Peakall & Smouse 2006). For each trait, association analyses via regression on individual SNP genotypes, a repeated gene substitution MIXED model and least square means (SAS Inst., Inc., Cary, NC, USA) were performed. In the first step any possible association between a polymorphism (SNP) within the *HSP90AB1* gene and a trait was analyzed using stepwise regression analysis. In the second step a gene substitution model was used to analyze breed specific effects and those of significant SNPs driven from the stepwise regression. The following model was applied:

$$Y_{ij}=\mu + B_i+\sum_k b_k (X_{ij})+ e_{ijk},$$

where Y_{ij} is the phenotypic value of heat associated traits; μ is the overall mean B_i is the fixed effect of ith breed; b_k is the regression coefficient on the number of copies of significant allele of *HSP90AB1* gene; k is the number of significant SNPs of the *HSP90AB1* gene. X_{ij} presents the copies of alleles of significant SNPs within *HSP90AB1*, and e_{ij} is the random error. The sire effect was not included in the statistical

model. Significance level of differences among genotype groups were determined at $p < 0.05$.

Results and Discussion

Polymorphism screen and population genetics parameters

Nine novel polymorphisms - SNP01 to SNP09 (three in exons, five in introns and one in the 3'UTR) - covering 5,664 bp of the bovine *HSP90AB1* were detected by comparative sequencing of 24 animals representing the six breeds. SNP07 led to a missense mutation (alanine to valine), the further SNPs proved to be silent. Allele and genotype frequencies are displayed in Table 5.2. Fixed allele frequencies were predominantly found in the HF group and the most balanced distribution of alleles over all data displayed the MT breed. A close to 1:1 ratio of alleles was only found for four SNPs (SNP04, SNP06, and SNP09 in MT, and SNP08 in WL). The calculated genetic heterozygosity based on allele frequencies was low in HF (0.071), but high in Thai native cattle (0.326 for MT of 0.307 for WL). The data suggest therefore a higher genetic diversity of Thai native cattle and in agreement with the study of Department of Livestock Development, Thailand (Boonyanuwat *et al.* 2005).

Table 5.2 Genotype and allele frequencies at the *HSP90AB1* locus in three cattle breeds

Regions [1]	SNPs	Genotypes	Genotype frequencies (n)				Alleles	Allele frequencies			
			HF	MT	WL	Pooled		HF	MT	WL	Pooled
Intron 8	1) g.4029G>C	CC	0	0	0	0	C	0	0	0.235	0.085
	(SNP01)	CG	0	0	0.471 (8)	0.170 (8)	G	1.000	1.000	0.765	0.915
		GG	1.000 (14)	1.000 (16)	0.529 (9)	0.830 (39)					
	2) g.4061G>A	AA	0	0.125 (2)	0.059 (1)	0.064 (3)	A	0	0.313	0.324	0.224
	(SNP02)	AG	0	0.375 (6)	0.529 (9)	0.319 (15)	G	1.000	0.688	0.676	0.776
		GG	1.000 (14)	0.500 (8)	0.412 (7)	0.617 (29)					
Intron 9	3) g.4338T>C	CC	0.643 (9)	0	0	0.191 (9)	C	0.821	0.156	0	0.298
	(SNP03)	CT	0.357 (5)	0.313 (5)	0	0.213 (10)	T	0.179	0.844	1.000	0.702
		TT	0	0.688 (11)	1.000 (17)	0.596 (28)					
Exon 10	4) g.4374T>G	GG	0	0.313 (5)	0.941 (16)	0.447 (21)	G	0.036	0.594	0.971	0.564
	(SNP04)	GT	0.071 (1)	0.563 (9)	0.059 (1)	0.234 (11)	T	0.964	0.406	0.029	0.436
		TT	0.929 (13)	0.125 (2)	0	0.340 (15)					
Intron 10	5) g.4730A>C	AA	1.000 (14)	1.000 (16)	0.588 (10)	0.851 (40)	A	1.000	1.000	0.794	0.925
	(SNP05)	AC	0	0	0.412 (7)	0.149 (7)	C	0	0	0.206	0.075
		CC	0	0	0	0					
Exon 11	6) g.5007T>C	CC	0	0.313 (5)	0.941 (16)	0.447 (21)	C	0.036	0.563	0.971	0.553
	(SNP06)	CT	0.071 (1)	0.500 (8)	0.059 (1)	0.213 (10)	T	0.964	0.438	0.029	0.447
		TT	0.929 (13)	0.188 (3)	0	0.340 (16)					
	7) g.5082C>T	CC	1.000 (14)	0.563 (9)	0.882 (15)	0.809 (38)	C	1.000	0.719	0.941	0.883
	(SNP07)	CT	0	0.313 (5)	0.118 (2)	0.149 (7)	T	0	0.281	0.059	0.117
		TT	0	0.125 (2)	0	0.043 (2)					
Intron 11	8) g.5248C>T	CC	0.929 (13)	0.563 (9)	0.353 (6)	0.596 (28)	C	0.964	0.781	0.588	0.766
	(SNP08)	CT	0.071 (1)	0.438 (7)	0.471 (8)	0.340 (16)	T	0.036	0.219	0.412	0.234
		TT	0	0	0.176 (3)	0.064 (3)					
3'UTR	9) g.5435T>C	CC	0	0.313 (5)	0.941 (16)	0.447 (21)	C	0.036	0.531	0.971	0.543
	(SNP09)	CT	0.071 (1)	0.438 (7)	0.059 (1)	0.191 (9)	T	0.964	0.469	0.029	0.457
		TT	0.929 (13)	0.250 (4)	0	0.362 (17)					

[1]Numbers refer to GenBank Acc. No. NW001494158

Physiological parameters and their associations with HSP90AB1 sequence variants

Associations between sequence variants within *HSP90AB1* and physiological parameters were analyzed. Earlier, others have considered RR, PCV, RT and HTC as parameters to evaluate the heat stress/tolerance of cattle (Beatty *et al.* 2006; Liu *et al.* 2010; Liu *et al.* 2011). We elaborated the parameters further to define the traits AM-RR (respiratory rate in the morning), PM-RR (respiratory rate in the afternoon), AM-PCV (blood pack cell volume in the morning), PM-PCV (blood pack cell volume in the afternoon), AM-RT (rectal temperature in the morning) and PM-RT (rectal temperature in the afternoon). In addition, we used all recorded observations to calculate average values for RR, PCV and RT (ARR, APCV and ART). During the experimental time an averaged hot and humid climate of 22 ºC and 94% RH in the morning increased to 34 ºC and 68% RH in the afternoon. During the extreme heat in the afternoon, RR and RT traits were elevated in animals of each of the breeds, whereas all traits corresponding to the PCV decreased compared to the morning values. MT and WL cattle were significantly superior in all physiological traits including the HTC compared to HF (98.38 and 96.85 compared to 95.28) (Table 5.4). Table 5.3 summarizes the effects of the SNPs on the physiological parameters under heat stress condition using the stepwise regression analysis. To determine which of the nine SNPs were associated with the traits, a forward stepwise regression analysis was conducted ($\alpha = 0.05$ for inclusion and 0.05 exclusion). For five (SNP03, SNP06, SNP07, SNP08, and SNP09) of the nine SNPs which have an effect on at least one of the ten traits was computed. SNP03 was the most frequently associated DNA variation (effects on eight of the ten traits) with as well the highest significance thresholds. Effects of miscellaneous SNPs on a trait were evident, except for the RR traits.

HSP90AB1 SNPs and RR traits

An increased RR is an important thermoregulatory response to heat stress. It aids in heat dissipation via evaporative cooling (Hammond *et al.* 1996; Beatty *et al.* 2006). Thus, a low RR may indicate an improved thermo tolerance. Using the stepwise regression model, we report associations between SNP03 and SNP06 on RR traits approved by $p <$

0.05 or better. The possession of one T allele at SNP06 increased the AM-RR by 3.24 times/min ($p < 0.0001$), whereas one copy of the T allele at SNP03 lowered the PM-RR (-2.68 times/min) as well as the ARR (-3.10 times/min). The allele frequency distributions indicate a high frequency of the detrimental SNP06 T allele (0.96) in HF, but elevated frequencies of the supportive T allele at SNP03 in the indigenous Thai cattle. Contrary to that the gene substitution model (Table 5.4) suggests that this observation is primarily breed-specific: WL has lower RR compared to MT resp. HF for all three traits that also differ significantly ($p < 0.05$) except for AM-RR. For this trait no significant differences between MT and WL do exist. In literature, there is no evidence that under physiological conditions the respiratory capacity to handle heat is superior in zebu cattle. The proportion of evaporation was roughly similar for Brahman, Holstein, Jersey and Brown Swiss. Heat stress, however, enhances the evaporative heat loss via respiration in European breeds (Seif *et al.* 1979; Gaughan *et al.* 1999; Gaughan *et al.* 2010) indicating more sophisticated heat loss mechanisms in less-adapted breeds to higher temperatures (Hansen 2004).

HSP90AB1 SNPs and PCV traits

SNP03 resp. SNP07 were significantly ($p < 0.001$ resp. $p < 0.05$) associated with the PCV traits. In any case, the presence of the T allele provided an advantage of 2.62 to 4.50 % (Table 3). In turn, the gene substitution model proved that again only the breed attributed significantly to effects on PCV traits. MT animals revealed the highest percentage of recorded PCV, whereas HF animals showed the lowest and WL represented medium values. The T allele frequencies are in fact not the highest in MT. Thus we assume that these two SNPs might act rather as markers than as causative sequence variations for PCV traits. Putative physiological differences of *Bos taurus* resp. *Bos indicus* cattle to continuous heat and humidity were investigated previously by Beatty et al. (2006). The authors propose that the increased water consumption under higher temperatures will lead to an increased total blood volume and a decrease in PCV. We did not measure the total blood volume, the water intake – and also not the water output as urine – to assure this observation, but conclude that MT animals consume less water to keep the homeostasis compared to the other two breeds.

HSP90AB1 SNPs and RT traits

Most associations between SNPs within *HSP90AB1* and traits were recorded for rectal temperature. These effects were highly significant, but at the same time also inconsistent as well. In total, nine putative SNP effects existed. The stepwise regression analysis revealed effects of the T allele on the trait AM-RT: SNP06 accounts for a temperature raise of 1.10 °C, and a decrease of 0.99 °C is at the same time caused by a T allele at SNP09. The T and C allele frequency at the loci SNP06 and SNP09 is identical in HF and WL, but, however, not the one's for SNPs 07 and 08 that are physically lying in between. As the SNP positions are only 428 bp away from each other, recombination events in this gene area are possible. The estimated regression coefficient decreased for the traits PM-RT resp. ART in the presence of a T allele at SNP03 by -0.28 (p = 0.006) resp. by -0.11 (p = 0.019). A T allele at SNP07 was associated with a temperature increase of 0.23 (p = 0.012) resp. 0.16 (p = 0.009). Preferred HTCs are associated with the T allele at SNP03 and a detrimental effect on this trait comes from variant T at SNP08 (Table 5.3). The gene substitution model (Table 5.4) finally proved that only temperature traits are affected by both the breed and the investigated SNPs.

Table 5.3 The effect of different SNPs on heat stress response traits using stepwise regression analysis

Traits	Intercept [1]	SNPs [2,3]									
		SNP03 (g.4338T>C)		SNP06 (g.5007T>C)		SNP07 (g.5082C>T)		SNP08 (g.5248C>T)		SNP09 (g.5435T>C)	
		Estimate (SE)	P-value	Estimate (SE)	P-value	Estimate (SE)	P-value	Estimate (SE)	P-value	Estimate (SE)	P-value
AM-RR (times/min)	21.45	ns	ns	3.24 (0.67)	0.0001	ns	ns	ns	ns	ns	ns
PM-RR(times/min)	41.86	-2.68 (1.23)	0.035	ns	ns	ns	ns	ns	ns	ns	ns
ARR (times/min)	35.59	-3.10 (0.83)	0.0006	ns	ns	ns	ns	ns	ns	ns	ns
AM-PCV (%)	26.96	4.59 (0.85)	0.0001	ns	ns	3.12 (1.31)	0.022	ns	ns	ns	ns
PM-PCV (%)	24.70	3.10 (0.74)	0.0001	ns	ns	2.62 (1.14)	0.027	ns	ns	ns	ns
APCV (%)	26.76	3.86 (0.76)	0.0001	ns	ns	2.87 (1.18)	0.019	ns	ns	ns	ns
AM-RT (ºC)	37.96	ns	ns	1.10 (0.28)	0.0004	ns	ns	ns	ns	-0.99 (0.28)	0.001
PM-RT (ºC)	39.60	-0.28 (0.09)	0.006	ns	ns	ns	ns	0.23 (0.09)	0.012	-0.18 (0.09)	0.043
ART (ºC)	38.69	-0.11 (0.04)	0.019	ns	ns	ns	ns	0.16 (0.06)	0.009	ns	ns
HTC	96.10	1.09 (0.44)	0.019	ns	ns	ns	ns	-1.57 (0.57)	0.009	ns	ns

[1] Intercept is for all five SNPs the trait mean of the genotype CC. The estimates account for the presence of one copy of the T allele. Thus the estimate has to be doubled for TT gentoypes. [2] To determine which combination of the nine genotype SNPs were independently associated with different heat tolerant traits, a forward stepwise regression analysis was conducted ($\alpha = 0.05$ for inclusion and 0.05 exclusion) with each SNP coded with different genotypes (1^{st} = CC, 2^{nd} = CT and 3^{rd} = TT), ns = not significant. [3] Regression coefficients are estimated by considering all SNPs in model (SNP01 to SNP09).

Do HSP90AB1 SNPs contribute to heat stress/heat tolerance in Thai cattle breeds?

Thailand is located on the Indi-China peninsula. The climate is monsoonal tropical that remains hot and humid throughout the year. The average temperature is about 29 ºC, ranking in Bangkok (capital city) from 35 ºC in April to 17 ºC in December (MFA 2011). WL and the MT cattle are the most prominent native cattle breeds in Northern Thailand. They are rather fertile animals, tolerant towards a poor food quality and also towards internal and external parasites (Rattanaronchart 1998). The breeds are well adapted to the environment, but there were very few studies to prove this both with phenotypic and genetic data. In no case, the values of the investigated physical parameters were pathological, but the data clearly underline a superior performance of MT and WL compared to the *Bos taurus* individuals. There are several physiological mechanisms to cope with heat stress (i.e. sweating, high respiratory rate, rising rectal temperature above critical thresholds, increased water consumption, reduced metabolic rate, and a decreased dry matter intake) that at the same time reveal a negative impact on the production and reproduction performance of the cattle (West 2003; Hansen 2004; Beatty *et al.* 2006). It is well described that all of these physiological responses are substantially enhanced in *Bos taurus* compared to *Bos indicus* (Hammond *et al.* 1996; Collier *et al.* 2008). In addition, there is also ample evidence that the basal metabolic rate of *Bos indicus* is generally lower compared to *Bos taurus* (Gaughan *et al.* 1999; Hansen 2004; Gaughan *et al.* 2010). Clearly, low metabolic rates are consequences of reduced or low performance traits such as growth rate and milk yield. Thus low producing cattle (livestock) reveal an increased heat tolerance (Reid *et al.* 1991).

Table 5.4 Least squares means for different breed, regression coefficient for different SNP-genotypes and level of significance for different physical parameters on heat stress response traits[1]

Traits	Breeds[2]			SNPs [3,4]				
	HF	MT	WL	SNP03	SNP06	SNP07	SNP08	SNP09
AM-RR (time/min)	28.95 (1.01)[a]	23.85 (0.95)[b]	21.05 (0.92)[b]	nd	0.51 (1.20)	nd	nd	nd
PM-RR(time/min)	39.73 (1.76)[a]	40.20 (1.65)[a]	34.76 (1.60)[b]	-4.56 (2.49)	nd	nd	nd	nd
ARR (time/min)	34.34 (1.19)[a]	32.03 (1.11)[a]	27.92 (1.08)[b]	-2.31 (1.71)	nd	nd	nd	nd
AM-PCV (%)	26.53 (0.74)[a]	39.53 (0.80)[b]	35.34 (0.78)[c]	-0.34 (1.27)	nd	0.42 (1.06)	nd	nd
PM-PCV (%)	26.08 (0.71)[a]	36.44 (0.66)[b]	31.77 (0.64)[c]	-0.65 (1.07)	nd	0.03 (0.87)	nd	nd
APCV (%)	26.27 (0.72)[a]	37.98 (0.67)[b]	33.55 (0.65)[c]	-0.48 (1.06)	nd	0.23 (0.88)	nd	nd
AM-RT (ºC)	38.20 (0.11)[a]	37.95 (0.07)[a]	37.99 (0.10)[a]	nd	**0.92** (0.30)	nd	nd	**-0.90** (0.28)
PM-RT (ºC)	39.07 (0.15)[a]	39.02 (0.08)[a]	39.32 (0.11)[a]	**-0.26** (0.12)	nd	nd	**0.22** (0.08)	-0.08 (0.10)
ART (ºC)	38.77 (0.55)[a]	38.46 (0.05)[b]	38.61 (0.05)[ab]	0.01 (0.07)	nd	nd	**0.13** (0.05)	nd
HTC	95.28 (0.54)[a]	98.38 (0.47)[b]	96.85 (0.49)[ab]	-0.07 (0.76)	nd	nd	**-1.33** (0.53)	nd
	ANOVA significance level [P (F)]							
	Breeds			SNPs				
	Type I[4]	Type III		SNP03	SNP06	SNP07	SNP08	SNP09
AM-RR	<0.0001	0.041		nd	0.681	nd	nd	nd
PM-RR	0.036	0.077		0.075	nd	nd	nd	nd
ARR	<0.0001	0.123		0.183	nd	nd	nd	nd
AM-PCV	<0.0001	<0.0001		0.783	nd	0.688	nd	nd
PM-PCV	<0.0001	<0.0001		0.541	nd	0.973	nd	nd
APCV	<0.0001	<0.0001		0.648	nd	0.797	nd	nd
AM-RT	0.006	0.203		nd	**0.004**	nd	nd	**0.003**
PM-RT	0.005	0.089		**0.049**	nd	nd	**0.016**	0.458
ART	0.002	0.008		0.915	nd	nd	**0.017**	nd
HTC	0.002	0.008		0.926	nd	nd	**0.017**	nd

[a-b] Means within a factor with different superscripts differ significantly ($p < 0.05$). [1]Statistical analysis carried out using breed as fixed class variable and only fixed significant SNPs derived from stepwise regression analysis. [2]Ls-Means with standard error (SE) are estimated using statistical models by considering only significant factors. [3]Regression coefficients with standard error (SE) are estimated by considering all variables in the model; nd = not included in model due to non significance in stepwise regression analyses, bold is a significant effect of SNPs on Type III analysis.-[4]The variable breed placed always in the first position in model.

Conclusions

The 'trait' heat tolerance is a quantitative trait (Gaughan *et al.* 2010; Li *et al.* 2010; Liu *et al.* 2011). Several studies aimed to find the link between phenotypes and genotypes. A SNP at nucleotide position 2789 within *ATP1A1* mRNA is known to be associated with heat tolerance traits in dairy cows (Liu *et al.* 2010; Liu *et al.* 2011). Effects of the SNP g.1524G>A, g.3494T>C and g.6601G>A within *HSP70A1A* affects thermo tolerance in Chinese Holstein cattle (Li *et al.* 2010). However, there have been no reports of genetic variations in bovine HSP90 genes and heat tolerance. The association analysis using a stepwise regression revealed that the T allele at SNP g.4338T>C improved the heat tolerance ($p < 0.05$) of the animals. Allele T was exclusively found in WL animals and to 84% in MT. HF cattle revealed an allele frequency of only 18%. The study indicates breed specific physiological responses to heat stress. Here, polymorphisms within *HSP90AB1* were not causative for the physiological responses, however, the results propose that this gene is an attractive candidate for heat tolerance, and should at least be used as a genetic marker to select appropriate breeds for hot climates. However, to finally cope well with heat stress further factors including housing, nutrition, health status, age, and body condition have to be considered (Gaughan *et al.* 2010).

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CHAPTER 6

General Discussion and Conclusions

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1 General Discussion

The overall goals of this study were to gain genetic information on the molecular level that is indispensable to conserve indigenous Thai pigs and cattle breeds as well as to define their potential as genetic resources. There were several specific aims of this study: (1) The investigation of the mtDNA composition and the determination of genetic diversity in pig populations indigenous to Northern Thailand (Chapter 2). (2) The assessment of the phylogeny of Thai indigenous pigs, the comparison with further Asian and European pigs and the determination of their origin of domestication (Chapter 3). (3) The comparison of the genetic background of Thai indigenous pigs with commercial pigs used for meat production in Thailand and with selected Chinese pig breeds (Chapter 4). (4) The searching for sequence polymorphisms within the bovine *HSP90AB1,* to record physiological responses against heat stress and to describe putative associations between them in three cattle breeds used in Thailand (Chapter 5).

As reviewed in Chapter 2, indigenous livestock in Thailand have played an important role in smallholder farms and local populations for long time ago as they adapt well to hot and humid climates, tolerate low quality feed, and are probably resistant to some disease and internal parasites. They also contribute to the maintenance of cultural traditions (Rattanaronchart 1994; 1998a; Nakai 2008b; a; DAD-IS 2010). Moreover, their genetic potential could be a reservoir of genetic variation, which will be used as an important genetic resource. However, nowadays, livestock production of Thailand is growing very quickly and has been shifting from backyard animals and integrated crop-livestock farming systems to industrial livestock farming enterprises (FAO 2002; DLD 2010). Exotic livestock was imported to improve the production performance for economically important traits. Indigenous livestock has therefore gradually been used for crossbreds and was finally completely replaced by the exotic commercial breeds. These breeding strategies will lead to the threatening risk of losing genetic identity and diversity of indigenous breeds. Although Thai indigenous livestock have been described by the domestic animal diversity information system

(DAD-IS 2010) of the Food and Agriculture Organization (FAO), it is nevertheless difficult to determine real characteristics specific for each breed. Very few studies have been conducted on their genetic information, especially in pigs (Rattanaronchart 1994; Charoensook *et al.* 2009a; Charoensook *et al.* 2009b) and cattle (Rattanaronchart 1998a; Boonyanuwat *et al.* 2005). The need to conserve and to utilize existing genetic diversity of indigenous livestock has become a concept of highest importance worldwide. Studies on genetics, the development of economic traits and the preservation of indigenous breeds are crucial to defining and registering genetic resources (Rege & Okeyo 2006). Molecular markers have proven to be the best tools available to estimate genetic diversity, to assess phylogenetic relationships and thus to ensure sustainable animal breeding. In this thesis, mtDNA, microsatellites and Single Nucleotide Polymorphism (SNP) analysis focusing on indigenous pigs and cattle were discussed.

Thai indigenous pigs are predominantly raised by communities in the Northern part with almost half of the country's indigenous pig population. However, small pig populations without any scrutinized breeding programmes are always at risk of losing genetic identity and diversity (Charoensook *et al.* 2009a; Charoensook *et al.* 2009b). The investigation of genetic structure, genetic diversity and phylogenetic relatiionship using mtDNA and microsatellites are presented in Chapter 2, 3 and 4. MtDNA is maternally inherited without any recombination evidence. Therefore, the number of nucleotide differences between mitochondrial genomes directly reflects the genetic distance that separates them. Secondly, it mutates 5-10 times more frequently than nuclear DNA, thus allowing the study of the divergence between wild and domestic populations under the short time scale of domestication (Toro *et al.* 2009). Thus, the complete mtDNA control region was comparatively sequenced to determine the degree of shared haplotypes, the population structure and the phylogenetic relationships within pig populations. For that, we collected 72 Thai native pigs and 11 Thai wild boars in five regions (i.e. Mae Hongson, Southern and Northern part of Chiang Mai, Chiang Rai and Uttaradit provinces) of Northern Thailand. In total 36 nucleotide polymorphisms were found in the 83 investigated animals accounting for 24 different mtDNA haplotypes termed TNH01 to TNH20 and TWH01 to TWH04.

Phylogenetic analyses were performed by several methods. The result showed that the phylogenetic tree has separated into two main clades: i.e. a European (E) and an

Asian (A) clade with further Asian subclades (AS1, AS2 and THG) and a single not incorporated haplotype representing the Taiwanese Lanyu pig. Twenty-three of the 24 Thai haplotypes arranged randomly in AS1 that is similar to the D2 cluster. This cluster is widely distributed among domestic Chinese and related pigs as well as the East Asian wild boars (Larson *et al.* 2005; Larson *et al.* 2007). Finally, eight of the 23 Thai haplotypes (i.e. 27 of the 78 tested individuals or a total of 34.6%) in clade A recapitulated another major cluster (denoted Thai haplogroup = THG) that was supported by a high bootstrap value. The assignment of the 15 porcine Thai haplotypes to cluster AS1, supports the hypothesis of a shared common ancestors with the Chinese domestic pigs, but the formation of the separate THG is also most putatively an indication for a further independent domestication event in Southeast Asia (SEA) in the past (Larson *et al.* 2010).

The average pairwise distances of 0.0136 ± 0.0029 (between AS2 and THG), of 0.0109 ± 0.0023 (between AS2 and AS1) and of 0.0084 ± 0.0023 (between THG and AS1) resulted in estimates for the time since divergence of 90,000 to 496,000 years between mtDNA clade AS2 and clade THG, 72,000 to 397,000 years between clade AS2 and clade AS1, and 56,000 to 306,000 years between clade THG and clade AS1. The data imply that THG and AS1 diverged from the AS2 clade, but also that AS1 is evolutionary older than THG. The data support even more the hypothesis that anchestoral animals of both AS2 and AS1 contributed to establish THG. Tanaka *et al.* (2008) have proposed that mtDNA haplotypes specific for pigs indigenous to the mountainous areas of Bhutan, Cambodia, Laos and Myanmar constitute a clade of their own (MTSEA). As the majority of these countries share borders with Thailand. The star-like pattern network demonstrates that all MTSEA haplotypes – except haplotypes H29 (Bhutan pigs only) and H33 (Myanmar pigs only) – are integrated into the THG haplogroup or vice versa. The MTSEA-THG haplogroup is shown in Figure 3.2.

Recently, Larson *et al.* (2010) have identified MTSEA as a mixed clade 3 of wild and domestic samples (MC3) that were found almost exclusively in the Indo-Burma Biodiversity Hotspot (IBBH) that includes Laos, Cambodia, Myanmar and Thailand to the Kra Isthmus. The high degree of genetic diversity within the IBBH is most probably the consequence of demographic expansions of agricultural populations and thus domestic pigs into this area (Larson *et al.* 2005; Larson *et al.* 2007; Larson *et al.* 2010).

The MTSEA-THG haplogroup also displays the history of Thailand, as it represents only haplotypes that are indigenous to provinces that previously belonged to the former Thai Kingdom (Siam). Genetic exchange between these provinces was always possible because of transport routes (i.e. the Mekong river) and migration of people between provinces.

Genetic diversity at the nuclear level within and between Thai indigenous pig populations was assessed by microsatellite analysis in Chapter 4. Microsatellites have been the markers of choice to study genetic variation in recent years. Based upon sites in which the same short sequence is repeated multiple times, they present a high mutation rate and have a co-dominant nature. This makes them appropriate for the study of both within and between breed genetic diversity. Studies to document the genetic diversity of indigenous pig breeds and to decipher phylogenetic relationships with further breeds or lines using microsatellite markers are numerous. Despite that the ISAG/FAO Standing Committee for biodiversity has recommended a panel of 27 pre-selected microsatellites (FAO 2004) this has been used only in few times. In our study, we used 24 of the recommended 27 microsatellites. Three STS-markers S0178, S0228 and SW24 were replaced in our survey by markers S0120 and SW1031 to cover all porcine chromosomes including the sex chromosomes. This study point out that Thai native pigs and Thai wild boars show advantageous H_E and H_O compared to the commercial pigs, but not always to the crossbreds that revealed a surplus of heterozygous animals ($H_O > H_E$). A high heterozygosity must be attributed to heterosis, and at the same time to a marginal degree of inbreeding effects. The calculated inbreeding coefficient (*F*) was zero in the crossbreds and also low in all of the commercial breeds (Table 4.3). The sophisticated breeding programs in commercial pigs should have led to their negligible degree of inbreeding. Unexpectedly high was instead the inbreeding in individual of the Southern part of Chiang Mai province (0.139), Chiang Rai (0.105) and the Thai wild boars (0.100). Assuming no previous inbreeding between any parents, a *F* of at least 0.125 is expected if either grandfather/granddaughter (grandmother/grandson), alternatively half-brother/half-sister or uncle/niece (aunt/nephew) mating occurred. We cannot rule that out as animals of these populations were kept in small villages for generations (SCM and CR) or were caught and kept in captivity as in the case for the TWB.

The study proposed a close relationship between Thai native and Chinese pigs in both mtDNA and nuclear DNA level. At the same time both populations are distinctly different from European lineages. Any genetic differentiation among different breeds or populations was probably due to selection, drift, and local inbreeding effects. On the other hand, the close genetic relationship between some Thai native pigs and the crossbreds could be a direct effect of a genetic introgression from European pigs. In 1957, the Department of Livestock Development (DLD) at the Ministry of Agriculture, under the guidance of FAO, has started to import European pig breeds into Thailand and has promoted to raise and sale them in local areas. Thus, native pigs have been increasingly mated with European breeds (Rattanaronchart 1994).

The discovery of unique signatures in mtDNA control region at position 24 (nucleotide A), position 183 (nucleotide C) and at position 896 (nucleotide G) in all members of the THG will be a benefit to the identification of them from all other known porcine haplotypes. Moreover, the private microsatellite alleles that were reported here for native pig populations in Uttaradit (S0155, SW240, S0002), Northern part of Chiang Mai (S0002, S0225), Chiang Rai (SW911), Southern part of Chiang Mai (S0068), and Thai wild boars (S0227, SW122) will provide additional information to genetically describe the uniqueness of the Thai indigenous pig population. The present study has revealed that some of the local pig populations show signs of genetic erosion, clearly indicating that urgent measures of conservation and sustainable management of their gene pool must be undertaken. The all provided genetic information is therefore a benefit for both conservation purposes as well as the utilization of them as an important genetic resource to improve future pig production in Thailand.

White Lamphun and the Mountain cattle are the most prominent native cattle breeds in Northern Thailand. They are rather fertile animals, tolerant towards a poor food quality and also towards internal and external parasites (Rattanaronchart 1998b). The breeds are well adapted to the environment, but there were very few studies to prove this both with phenotypic and genetic data. In this study, the polymorphism within the bovine *HSP90AB1* (Chapter 5) showed high degree of genetic diversity in Thai indigenous cattle. The calculated genetic heterozygosity based on allele frequencies was low in HF (0.071), but high in Thai native cattle (0.326 for MT of 0.307 for WL). These results are in agreement with the study of Department of Livestock Development,

Thailand (Boonyanuwat *et al.* 2005). In our study, nine novel SNPs were identified, i.e. three in exons 10 and 11, five in introns 8, 9, 10, 11, and one was located in the 3'UTR (Table 5.2). The exon 11 SNP g.5082 C>T led to a mis-sense mutation (alanine to valine), the further SNPS proved to be silent. Fixed allele frequencies were predominantly found in the HF group and the most balanced distribution of alleles over all data displayed the MT breed. A close to 1:1 ratio of alleles was only found for four SNPs (g.4374T>G, g.4730A>G, and g.5435T>C in MT, and g.5248C>T in WL) as shown in Table 5.2.

Thailand is a tropical country and lies in the hot and humid climatic zones of the world. The environmental heat, resp. the heat stress, is most detrimental to cattle production and welfare which can be visible, for example, by a hindrance of feed consumption, a decreased milk production and a limited reproduction performance. Heat shock proteins like the Hsp90 gene act as molecular chaperones that have preferentially been transcribed in response to severe perturbations of the cellular homeostasis such as heat stress (environmental temperatures). Here the traits respiration rate (RR), rectal temperature (RT), pack cell volume (PCV), and the individual heat tolerance coefficient (HTC) were recorded as physiological responses on heat stress. Chapter 5 shows the study of physiological responses against heat stress and the descriptions of putative associations between bovine *HSP90AB1* gene in three cattle breeds used in Thailand. The results indicate that Mountain cattle and White Lamphun cattle were significantly superior in all physiological traits compared to Holstein Friesian crossbreds (98.38 and 96.85 compared to 95.28) (Table 5.4), including the heat tolerant coefficient (HTC) which have been calculated based on rectal temperature. Most associations between SNPs within *HSP90AB1* and traits were recorded for this trait. These effects were highly significant, but at the same time also inconsistent as well.

An increased respiratory rate is an important thermoregulatory response to heat stress. It aids in heat dissipation via evaporative cooling (Hammond *et al.* 1996; Beatty *et al.* 2006). Thus, a low RR may indicate an improved thermo tolerance. The gene substitution model (Table 5.4) suggests that this observation is primarily breed-specific: WL has lower RR compared to MT resp. HF for all three traits that also differ significantly ($p < 0.05$) except for respiratory rate in the morning. For this trait no

significant differences between MT and WL do exist. In literature, there is no evidence that under physiological conditions the respiratory capacity to handle heat is superior in zebu cattle. The proportion of evaporation was roughly similar for Brahman, Holstein, Jersey and Brown Swiss. Heat stress, however, enhances the evaporative heat loss via respiration in European breeds (Seif *et al.* 1979; Gaughan *et al.* 1999; Gaughan *et al.* 2010) indicating more sophisticated heat loss mechanisms in less-adapted breeds to higher temperatures (Hansen 2004). Putative physiological differences of *Bos taurus* resp. *Bos indicus* cattle to continuous heat and humidity were investigated previously by Beatty et al. (2006). The authors propose that the increased water consumption under higher temperatures will lead to an increased total blood volume and a decrease in pack cell volume. We did not measure the total blood volume, the water intake – and also not the water output as urine – to assure this observation, but conclude that MT animals consume less water to keep the homeostasis compared to the other two breeds.

Heat tolerance is a quantitative trait (Gaughan *et al.* 2010; Li *et al.* 2010; Liu *et al.* 2011). Several studies aimed to find the link between phenotypes and genotypes. Effects of the SNP g.1524G>A, g.3494T>C and g.6601G>A within *HSP70A1A* affects thermo tolerance in Chinese Holstein cattle (Li *et al.* 2010). However, there have been no reports of genetic variations in bovine HSP90 genes and heat tolerance. The association analysis using a stepwise regression revealed that the T allele at SNP g.4338T>C improved the heat tolerance ($p < 0.05$) of the animals. Allele T was exclusively found in WL animals and to 84% in MT. HF cattle revealed an allele frequency of only 18%. The study indicates breed specific physiological responses to heat stress. Here, polymorphisms within *HSP90AB1* were not causative for the physiological responses, however, the results propose that this gene is an attractive candidate for heat tolerance, and should at least be used as a genetic marker to select appropriate breeds for hot climates.

2 Conclusions and Recommendations

Based on the result of this study several conclusions and recommendations can be drawn:

(1) Thai native pigs are highly polymorphic and are closely related with Thai wild boars at the mtDNA and nuclear level, but are also distinctly separated from them.

(2) The genetic background and genetic diversity of the Thai indigenous pig populations revealed a close genetic relationship between them and Chinese pigs as well as the genetic introgression from European breeds.

(3) The assignment of the 15 porcine Thai mtDNA haplotypes to cluster AS1, supports the hypothesis of a shared common ancestors with the Chinese domestic pigs, but the formation of the separate MTSEA-THG clade is also most putatively an indication for a further independent domestication event in Southeast Asia (SEA) in the past.

(4) All members of the MTSEA-THG haplogroup have revealed unique signatures at position 24 (nucleotide A) and at position 183 (nucleotide C) that differentiate them from all other known porcine haplotypes.

(5) Some of the local pig populations show signs of genetic erosion, clearly indicating that urgent measures of conservation and sustainable management of their gene pool must be undertaken.

(6) Polymorphism within the bovine *HSP90AB1* gene demonstrated that Thai native cattle have high degree of genetic diversity.

(7) The association analysis revealed that the T allele at SNP g.4338T>C within intron 3 improved the heat tolerance, which was exclusively found in White Lamphun cattle and to 84% in Mountain cattle.

(8) We propose that polymorphisms within *HSP90AB1* should at least be used as genetic markers to select appropriate breeds for hot climates.

(9) The indigenous livestock are raised using few input but they still generate their products and by-products for house hold needs. In relation to

biodiversity, indigenous livestock seem to be a reservoir of genes which could be a benefit for the future use.

(10) It could be assumed that genetic diversity of indigenous livestock in Thailand is a product of different breeding programmes and farming systems.

(11) The primary focus of the study was to evaluate the genetic of indigenous livestock in Northern Thailand that are the main genetics resource of native pigs in this country. However, the result should be confirmed by the large scale investigations in the other part of Thailand.

(12) The mtDNA and microsatellite analysis of Thai indigenous cattle should be conducted to better understand their genetic background and origin.

(13) The provided genetic information is therefore a benefit for both conservation purposes as well as the utilization of them as an important genetic resource to improve future livestock production in Thailand.

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ZUSAMMENFASSUNG

In den letzten Jahren hat sich die Nutztierhaltung in Thailand von einer extensiven zu einer stark industrialisierten Tierhaltung hin entwickelt. Zu diesem Zwecke wurden ‚exotische' Rassen importiert, um so die Produktion ökonomisch wichtiger Merkmale zu steigern. Einheimische Rassen/Populationen wurden daher vorrangig für Kreuzungszuchten benutzt und letztendlich komplett durch exotische kommerzielle Zuchten ersetzt. Derartige Züchtungsstrategien widersprechen jedoch dem Konzept der Nachhaltigkeit und des Ressourcenmanagements. Es besteht das Risiko des Verlusts der genetischen Einzigartigkeit und der Diversität der einheimischen Rassen/Populationen. Aus diesen Gründen war das Ziel dieser Untersuchung die molekulargenetische Charakterisierung thailändischer Nutztiere (Schweine und Rinder), um deren Potential als genetische Ressource zu beschreiben. Die Arbeitsziele waren folgende: (1) Die Bestimmung der genetischen Diversität anhand der mtDNA einheimischer Schweinepopulationen in Nord-Thailand; (2) Die Untersuchung der Phylogenie thailändischer Schweinerassen/Schweinepopulationen, um sie mit asiatischen und europäischen Schweinerassen zu vergleichen. Zudem sollte für die thailändischen Rassen/Populationen Ort und Zeit ihrer Domestikation ermittelt werden; (3) Der Vergleich des genetischen Hintergrunds einheimischer und kommerzieller Schweineherkünfte, die für die Fleischproduktion in Thailand genutzt werden mit ausgewählten chinesischen Schweinerassen/Schweinepopulationen (z.B. Jiangquhai, Luchuan, Minzhu, Rongchang, Yujiang und Tibetan), (4) Die Darstellung von Sequenzvarianten des bovinen HSP90AB1-Gens, um mögliche Assoziationen zu physiologischen Parametern als Antwort auf Hitzestress bei drei thailändischen Rinderrassen zu beschreiben.

Die vollständige mtDNA-Kontrollregion (1264-1324 bp, abhängig vom Individuum) wurde vergleichend sequenziert. Anhand dieser wurden Haplotypen, die Populationsstruktur und die phylogenetische Verwandtschaft innerhalb der thailändischen Schweinepopulation bestimmt. Dafür wurden Proben von 72 einheimischen Schweineherkünften und 11 einheimischen Wildschweinen in fünf verschiedenen Regionen Nord-Thailands (Mae Hongson, südlicher und nördlicher Teil

von Chiang Mai, Chiang Rai und Uttaradit) gesammelt. Insgesamt führten 36 Nukleotidvariationen zur Bildung von 24 Haplotypen (TNH01 zu TNH20 und TWH01 zu TWH04). Der phylogenetische Baum trennte sich in einen europäischen Zweig (E) und einen asiatischen Zweig (A) auf. Der asiatische Zweig wies weitere Verästelungen auf (AS1, AS2 und THG). Dreiundzwanzig der 24 Haplotypen (außer TNH01) konnten dem asiatischen Zweig des Baumes zugeordnet werden. Acht dieser Haplotypen wurden zu einem weiteren Hauptcluster von Haplotypen (THG) zusammengefasst. Die durchschnittlichen paarweisen Distanzen von 0,0136 ± 0,0029 (zwischen AS2 und THG), von 0,0109 ± 0,0023 (zwischen AS2 und AS1) und von 0,0084 ± 0,0023 (zwischen THG und AS1) weisen darauf hin, dass eine Zeitspanne von ca. 90.000-496.000 Jahren zwischen der Trennung der beiden Äste AS2 und THG liegt. Zwischen AS2 und AS1 liegt eine Zeitspanne von etwa 72.000-397.000 Jahren und zwischen THG und AS1 eine Spanne von ungefähr 56.000-306.000 Jahren vor. Die Daten weisen daraufhin, dass THG und AS1 sich vom AS2-Zweig abgespalten haben, aber auch, dass AS1 evolutionär älter ist als THG. Zusammenfassend lässt dies vermuten, dass einheimische thailändische Schweineherkünfte eng verwandt mit thailändischen Wildschweinen sind, sich trotzdem aber auch deutlich unterscheiden. Beide lassen sich auf einen gemeinsamen asiatischen Vorfahren zurückführen.

Bei Berücksichtigung von lediglich 510 bp der sequenzierten mtDNA wurden alle THG-Haplotypen in den MTSEA-Zweig (alpiner und südostasiatischer Raum) integriert. Diese Haplogruppe wurde MTSEA-THG genannt. Vor kurzem wurde MTSEA in MC3 umbenannt. MC3 beinhaltet nur Signaturen von Schweinen, die über den sogenannten Indo-Burma Biodiverstäts-Hotspot (IBBH) verteilt sind. Der IBBH umschließt geographisch eine Region, die Thailand bis zum Kra Isthmus beinhaltet. Die Zuteilung der 23 porcinen Thai-Haplotypen zum AS1-Cluster unterstützt die Hypothese eines gemeinsamen Vorfahrens mit den chinesischen Hausschweinen. Dennoch ist der separierte MTSEA-THG-Zweig ein vermeintlicher Hinweis auf eine frühere unabhängige Domestikation in Südostasien (SEA). Die Haplotypen der Haplogruppe MTSEA-THG weisen einzigartige und bisher unbekannte Nukleotidsignaturen an den Positionen 24 (Nukleotid A) und 183 (Nukleotid C) auf, wodurch sie von allen anderen bekannten porcinen mtDNA-Haplotypen unterschieden und abgegrenzt werden können.

Der genetische Hintergrund und die genetische Diversität wurden am Untersuchungsmaterial auch anhand von 26 Mikrosatellitenmarker untersucht. Die thailändischen Schweineherkünfte zeigten eine hohe genetische Diversität, die sich in relativ hohen Werten für die effektive Heterozygotie (He; 0,71) und der effektiven Allelanzahl (Ne; 3,71) widerspiegeln. Weiterhin kann durch die genetische Distanz, die paarweise Anzahl verschiedener Allele, den Neighbour-Joining-Tree und die multidimensionale Analyse eine nahe genetische Verwandtschaft zwischen den thailändischen und den chinesischen Schweineherkünften nachgewiesen werden. Dennoch liegt auch eine genetische Introgression, die auf europäische kommerzielle Zuchten zurückzuführen ist, in thailändischen Schweinen vor. Die genetische Analyse zeigt deutlich, dass thailändische Schweinepopulationen einzigartige genetische Ressourcen darstellen.

Thailand ist ein tropisches Land und liegt in den heißen und feuchten Klimazonen. Die Umgebungshitze bzw. der Hitzestress ist vor allem für die Rinderproduktion von Nachteil. Dadurch kann das Wohlbefinden der Tiere beeinflusst werden, was mit verminderter Futteraufnahme, Rückgang der Milchleistung und einer limitierten Reproduktionsleistung einhergeht. Hitzeschockproteine werden als Regulatoren des Hitzestresses angesehen, die bevorzugt als Antwort auf die Störung des zellulären Gleichgewichts, transkribiert werden.

Aus diesem Grund werden die Merkmale Respirationsrate (RR), Rektaltemperatur (RT), Hämatokritwert (PCV) sowie der individuelle Hitzetoleranzkoeffizient als physiologische Antwort auf Hitzestress (Umwelttemperatur) in *Bos taurus* (Kreuzungszucht Holstein Friesian; HF) und *Bos indicus* (Thailändische Rinder: White Lamphun; WL und Mountain cattle; MT) aufgenommen. Siebenundvierzig äußerlich gesunde, nicht-laktierende Kühe wurden zufällig ausgewählt und auf dem Versuchsgut der Chiang Mai Universität in Thailand gehalten. Um Beobachtungen pro Tier zu erhalten, wurden RR und RT morgens (8:00 Uhr) und nachmittags (2:00 Uhr) für zwei Wochen pro Monat in vier aufeinanderfolgenden Monaten (September bis Dezember) gemessen. Während des Experimentes lag morgens die durchschnittliche Temperatur bei 22°C mit einer relativen Feuchtigkeit von 94%. Am Abend lag die Temperatur bei 34°C und 68% relativer Feuchtigkeit.

Polymorphismen innerhalb des Gens des Hitzeschockproteins 90-kDa-ß (*HSP90AB1*) wurden durch vergleichende Sequenzierung von *Bos taurus* und *Bos indicus* Individuen identifiziert. So konnten neun SNPs gefunden werden, d.h. drei in den Exons 10 und 11, fünf in den Introns 8, 9, 10 und 11 und einer im 3'UTR-Bereich des Gens. Der SNP g.5082 C>T in Exon 11 führt zu einem Aminosäurenaustausch (Alanin zu Valin). Die anderen SNPs sind stille Mutationen. Die berechnete genetische Heterozygotie, basierend auf den Allelfrequenzen, lässt eine höhere genetische Diversität thailändischer Rinder (MT=0,326 und WL=0,307) im Vergleich zu *Bos taurus* (HF=0,071) vermuten. Während der Zeit der extremen Hitze (am Nachmittag) wurden erhöhte Werte für RR und RT in allen drei Rassen gemessen, während der PCV hingegen abnahm. MT- und WL-Tiere waren in allen physiologischen Merkmalen den HF-Tieren überlegen. Die Assoziationsanalyse erklärt, dass das T-Allel am SNP g.4338 T>C innerhalb des Introns 9 die Hitzetoleranz der Tiere verbessert (p<0,05). Das T-Allel war zu 100% in allen WL- und zu 84% in den MT-Tieren nachweisbar, während die Frequenz bei HF-Rindern lediglich 18% betrug. Die vorliegende Untersuchung lässt eine rassespezifische physiologische Antwort auf Hitzestress erkennen. Polymorphismen innerhalb des *HSP90AB1* waren nicht ursächlich verantwortlich für die physiologische Antwort auf Hitzestress. Nichtsdestoweniger lassen die Ergebnisse dennoch erkennen, dass dieses Gen ein attraktiver Kandidat für Hitzetoleranz ist und als genetischer Marker für die Zucht von an heißen Klimaten angepassten Rassen verwendet werden kann.

APPENDICES

Appendix 1: Chemicals and Reagents

1.1 Chemicals

1) Absolute ethyl alcohol: Merck, USA
2) Acetic acid (glacial): Amersham Bioscience, Germany
3) Agarose powder (ultra pure): Biozyme, Germany
4) Boric acid: Roth, Germany
5) Bromophenol blue: Sigma, Germany
6) Dethyl pyrocabonate (DEPC): Roth, Germany
7) Dimethyl sulfoxide (DMSO): Sigma, Germany
8) di-Sodium hydrogen phosphate: Roth, Germany
9) Ethidium bromide: Roth, Germany
10) Ethylenediaminetetraacetic acid (EDTA): Roth, Germany
11) Formamide: Roth, Germany
12) Hydrochloric acid (HCl): Roth, Germany
13) Isopropanol: Sigma, USA
14) N, N'- dimethyl-formamide: Roth, Germany
15) Magnesium chloride: Qiagen, Germany
16) Potasium dihydrogen phosphate: Roth, Germany
17) Sodium acetate: Sigma, USA
18) Sodium chloride: Roth, Germany
19) Sodium dodecyl sulphate (SDS): Roth, Germany
20) Sodium hydroxide (NaOH): Roth, Germany
21) Tris: Roth, Germany

1.2 Reagents and preparation

All solutions used in this investigation were prepared with deionized or demineralised water (ddH_2O or Millipore water). The pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

1) 11% Agarose gel with ethidium:

Agarose powder	1.0	g
1X TBE buffer	100.0	ml
Ethidium bromide	7.0	µl

2) Digestion buffer:

2M Nacl (116.9 mg/ml)	5.0	ml
1M Tris pH 8.0 (121.1 mg/ml)	5.0	ml
0.5M EDTA (186.1 mg/ml)	0.2	ml
Millipore Water added to	100.0	ml

3) 10 mM dNTPs solution:

100 mM dATP	0.1	ml
100 mM dGTP	0.1	ml
100 mM dCTP	0.1	ml
100 mM dTTP	0.1	ml
Millipore Water added to	1.0	ml

4) 0.5M EDTA pH 8.0:

EDTA (Na2.2H2O)	186.1	g
Millipore Water added to	1000.0	ml
Adjust to pH 8.0		

5) Ethidium bromide solution:

Ethidium bromide	1.0	g
Millipore Water	100.0	ml

6) Phosphate buffer saline (PBS) pH 7.4:

Sodium chloride	8766.0	mg
di-Sodium hydrogen phosphate	1495.0	mg
Potassium dihydrogen phosphate	204.0	mg
Potassium chloride	200.0	mg
Millipore Water added to	1000.0	ml
Adjust to pH 7.4		
Autoclave		

7) 3M Sodium acetate pH 5.2:

Sodium acetate (MW 82.03)	246.1	g
Millipore Water added to	1000.0	ml
Adjust to pH 5.2		

8) 2M Sodium chloride:

Sodium chloride (MW 58.44)	116.9	g
Millipore Water added to	1000.0	ml

9) 6M Sodium chloride:

Sodium chloride (MW 58.44)	350.6	g
Millipore Water added to	1000.0	ml

10) 9% Sodium chloride:

Sodium chloride (MW 58.44)	9.0	g
Millipore Water	100.0	ml

11) 10% Sodium dodecyl sulfate (SDS):

Sodium dodecyl sulfate	10.0	g
Millipore Water	100.0	ml

12) 1X TBE buffer:

10X TBE buffer	20.0	ml
Millipore Water added to	1000.0	ml

13) 10X TBE buffer:

Tris	108.0	g
Boric acid	55.0	g
0.5M EDTA (186.1 mg/ml)	2.0	ml
Millipore Water added to	1000.0	ml

14) TE buffer:

1M Tris pH 8.0 (121.1 mg/ml)	10.0	ml
0.5M EDTA pH 8.0 (186.1 mg/ml)	2.0	ml
Millipore Water added to	1000.0	ml

15) 1M Tris pH 8.0:

Tris-base	121.1	g
Millipore Water added to	1000.0	ml
Adjust to pH 8.0		

Appendix 2: Enzymes, Nucleotides and Kits

2.1 Enzymes

1) Proteinase K**:** Qiagen, Germany
2) Restriction Enzymes: New England Biolabs GmbH, Germany
3) *Taq*-DNA Polymerase**:** Qiagen, Germany
4) *Taq*-DNA Polymerase**:** Roche, Germany

2.2 Nucleotides

1) Deoxyribonucleotide triphosphate (dNTP): Roth, Germany
2) Oligonucleotides: MWG Biotech AG, Germany

2.3 Kits

1) BigDye^{TM} -Terminator Cycle Sequencing Ready Reaction Kit: Applied Biosystems, Germany
2) DNA ladder and loading buffer: Amersham Biosciences, Germany
3) DNA ladder mix: Fermentas GmbH, Germany
4) ExoSAP-IT PCR clean-up Kit: Affymetrix, Germany
5) GeneScan^{TM} -500 ROX^{TM} Size Standard: Applied Biosystems, UK
6) PURE *Taq* Ready-To-Go PCR Beads®: Amersham Biosciences, Germany
7) QIA-amp DNA mini Blood Kit: Qiagen, Germany
8) QIA-quick PCR Purification Kit: Qiagen, Germany

Appendix 3: Equipments

1) Analytical Balances TE214S/TE6101: Satorius GmbH, Germany
2) Autoclave Varioklav 75S: ThermoScientific GmbH, Germany
3) Automated DNA Analyzer (ABI-3100): Applied Biosystems, Germany
4) Automated Spectrophotometer (ND-1000): Nanodrop, Germany
5) Bioclave: Schütt Labortechnik, Germany
6) Centrifuge 5424/5415R/5417R: Eppendorf, Germany
7) Deep Freezer: Schütt Labortechnik, Germany
8) Dest.-water (Biocell): Millipore, Germany
9) Electrophoresis equipment sets for agarose gel: Bio-Rad, Germany
10) Functional micropipetter: Eppendorf, Germany
11) Gel documentation system: PEQLAB Biotechnologie GmbH, Germany
12) Heat block QBD 2: Grant Instruments, UK
13) Incubator Certomat BS 1: Sartorius, Germany
14) Magnetic mixer KMO 2: Janke und Klunkel, Germany
15) Magnetic-mixer RCT basic: Schütt Labortechnik, Germany
16) Megafuge 1.0 R: Thermo, Germany
17) Set of Micropipette (0.5 µl to 1,000 µl): Eppendorf, Germany
18) Set of Micropipette (0.5 µl to 1,000 µl): Gilson, USA
19) Multifuge 1 sR: Thermo, Germany
20) PCR gradient (T-gradient): Biometra, Germany
21) PCR thermocycler (T-3000): Biometra, Germany
22) pH meter PB 11: Sartorius, Germany
23) Power supply PowerPac: Bio-Rad, Germany
24) Refrigerator 4°/-20°: Siemens GmbH, Germany
25) Sorvall centrifuge RC-5B: Du Pont Instruments, Germany
26) Speed Vac: Schütt Labortechnik, Germany
27) UV-Transilluminator (312nm and 366 nm): Amersham Biosciences, Switzerland
28) Vortex Genie 2: Bender & Hobein, Germany
29) Water Bath: Gesellschaft für Labortechnik, Germany

Appendix 4: DNA extraction and qualification

4.1 Genomic DNA extraction

Whole genomic DNA was extracted from blood (white blood cells) or ear clip (tissue) samples by the so called salting out method applied from Sambrook *et al.* (1989) and Miller *et al.* (1988). The hair sample (root hair cells) was extracted by using the QIA-amp DNA Blood mini kit (Qiagen, Germany). The DNA extraction protocols were as follows:

4.1.1 DNA extraction protocol for blood samples

1) Centrifuge blood samples (5-10 ml) at 6,000 rpm for 15 min and discard supernatant of plasma.
2) Carefully transfer the buffy coat (white blood cells) to a clean 1.5 ml microcentrifuge tube by a pasteur pipette.
3) Resuspend the buffy coat with 1 ml of millipore water, shake vortex for 20 sec to lysis red blood cells.
4) Add 100 µl of 9% sodium chloride solution to get a physiological condition. Shake by vortex, centrifuge at 12,000 rpm for 10 min. Discard the supernatant (repeat steps (3) and (4) until the pellet is white).
5) Resuspend the pellet with 1 ml of PBS solution, centrifuge at 12,000 rpm for 10 min and discard the supernatant.
6) Resuspend the pellet with 800 µl of digestion buffer, add 10 µl of proteinase K solution (20 mg/ml) and mix by vortex. Add 50 µl of 10% SDS solution and gently mix by hand.
7) Incubate overnight at 55^{o}C in a shaking incubator.
8) Incubate at room temperature for about 5-10 min, and then add 500 µl of 6M sodium chloride solution, incubate again at room temperature for 5-10 min, and then centrifuge at 12,000 rpm for 15 min.
9) Put the supernatant of about 500 µl into a clean 1.5 ml microcentrifuge tube.
10) Add one-tenth volume of 3 M sodium acetate solution and an equal volume of isopropanol. Gently shake the sample until precipitation of DNA.

11) Wash the DNA three times with 80% ethanol (centrifuge at 12,000 rpm for 5 min) and dry at room temperature.
12) Dissolve the DNA with 50-100 µl of TE buffer (until the concentration of DNA) and keep it at 4°C.

4.1.2 DNA extraction protocol for ear clip samples

1) Wash the 1 cm^2 ear clip with 80% ethanol and further with PBS solution. Transfer the sample into a clean 1.5 ml microcentrifuge tube.
2) Add 10 µl of proteinase K solution (20 mg/ml) and 800 µl of digestion buffer, mix by vortex, add 50 µl of 10% SDS solution and gently mix by hand.
3) Incubate overnight at 55°C in a shaking incubator.
4) Incubate at room temperature for about 5-10 min. Add 500 µl of 6 M sodium chloride solution, incubate again at room temperature for 5-10 min, and then centrifuge at 12,000 rpm for 15 min.
5) Put the supernatant of about 500 µl into a clean 1.5 ml microcentrifuge tube.
6) Add one-tenth volume of 3 M sodium acetate solution and an equal volume of isopropanol. Gently shake the sample until precipitation of DNA.
7) Wash the DNA three times with 80% ethanol (centrifuge at 12,000 rpm for 5 min) and dry at room temperature.
8) Dissolve the DNA with 50-100 µl of TE buffer (until the concentration of DNA) and keep it at 4°C.

4.1.3 DNA extraction protocol for hair samples by QIA-amp DNA Blood mini kit

1) Cut the root hairs (about 20 hairs for one sample) and take the sample into a clean 1.5 ml microcentrifuge tube.
2) Add 200 µl Millipore water and add 200 µl AL buffer.
3) Add 50 µl of proteinase K solution (20 mg/ml) and centrifuge briefly.
4) Incubate at 56°C for 3 hr and warm up the AE buffer.
5) Add 200 µl of 96% ethanol to the sample and mix again by hand for 30 sec and centrifuge at 14,000 rpm for 2 min.

6) Transfer the supernatant of sample into the QIAmp spin-column and centrifuge at 8,000 rpm for 2 min and change the collection tube.
7) Add 500 µl of AW1 buffer and centrifuge at 8,000 rpm for 2 min and change the collection tube again.
8) Add 500 µl of AW2 buffer and centrifuge at 8,000 rpm for 2 min.
9) Place the QIAmp spin-column in a clean 1.5 ml microcentrifuge tube, add 100 µl of warm AE buffer, incubate at room temperature for 5 min and then centrifuge at 14,000 rpm for 2 min. The DNA solution will drop into a 1.5 ml microcentrifuge tube.

4.2 DNA qualification

The absorbance of the DNA solution was measured to determine the amount and quality of the DNA from the optical density (O.D.) using the ND-1000 automed spectrophotometer version 3.1 (Nanodrop, Germany). The ratio of absorbance at 260 and 280 nm is used to assess purity of DNA, a ratio of about 1.8 is generally accepted as pure for DNA. Ratios lower than 1.75 indicate that significant amounts of proteins remained in the prepared sample. Samples were generally stored at 4ºC until PCR reactions were finished and then frozen at -20ºC for long-term storage. Repeated freezing and thawing of samples was avoided.

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