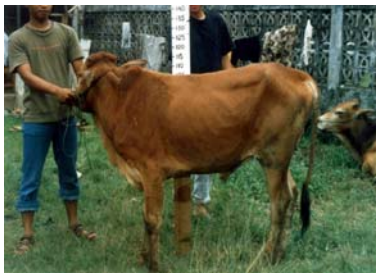
Institutionen för husdjursgenetik

Investigating genetic variability within specific indigenous Indonesian cattle breeds

by

Camilla Mannich Uggla




Photographs from Agus Nashri

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Examensarbete ingår som en obligatorisk del i utbildningen och syftar till att under handledning ge de studerande träning i att självständigt och på ett vetenskapligt sätt lösa en uppgift. Föreliggande uppsats är således ett elevarbete och dess innehåll, resultat och slutsatser bör bedömas mot denna bakgrund. Examensarbete på D-nivå i ämnet husdjursgenetik, 20 p (30 ECTS).

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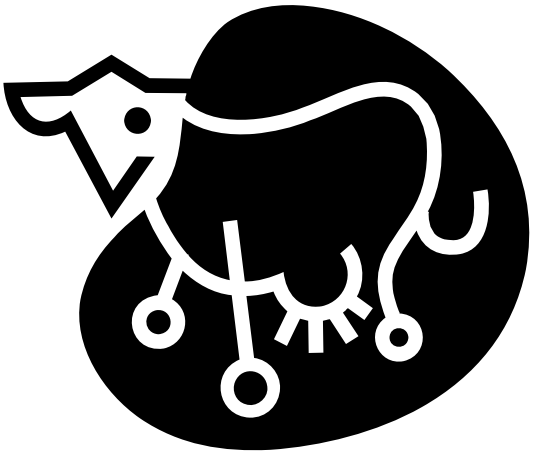

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Agrovoc: DNA, mitochondria, microsatellites

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Abstract

Evolution is the source of the huge variation between and within species worldwide. All species are related to each other through a common origin and new species will develop in the future from the existing species nowadays. Modern biology defines evolution as the differences in allele frequencies over time and these differences can be used for characterization of populations. In this study the genetic variability between different indigenous cattle breeds in Indonesia were investigated. For this purpose, genomic and mitochondrial DNA from160 cattle from the Aceh province, 10 Bali cattle, two Madura cattle, two Pesisir cattle and two Ongole descendent were used. For the analysis of the species origin, 20 microsatellite markers in the genome were used and tracing of maternal lineage and history of the herd was performed by analysis of the mitochondrial DNA (mtDNA), D-loop. The microsatellites were polymorphic in all breeds, ranging from two to twelve detected alleles with the exception for the Banteng where HEL 13 and INRA35 were monomorphic. Mean heterozygosity computed across the 16 loci for each breed ranged between 0, 3924 (Banteng) and 0, 7860 (Ongole). The inbreeding coefficient F_{IS} which indicates within breed genetic variation ranged from 0, 08851 (Pesisir) to 0, 14251 (Madura). In the Neighbor Joining tree, genetic relationships among the mtDNA sequences revealed that all samples with different origin clustered together, *Bos taurus, Bos indicus, Bos banteng.* In the median joining network the sequences with *Bos indicus* origin grouped into two star-like clusters with predominant haplotypes in the centres. The two clusters are separated by four mutations and one haplotype unique for the Aceh cattle. In summary, Banteng, the endangered ancestor of Bali cattle, exhibited low genetic variation compared with the other breeds in the study. The Aceh cattle showed highest level of genetic variation and is probably a breed which has been created using a large part of the zebu population as breeding animals. In the median joining network a unique haplogroup for the Aceh breed was revealed and with that a possible independent domestication process for the zebu cattle in Indonesia.

Sammanfattning

Evolutionen är orsaken till den stora variationen mellan och inom arter man kan se världen över. Alla arter är besläktade med varandra genom ett gemensamt ursprung och nya arter kommer att utvecklas i framtiden från arter befintliga idag. Modern biologi definierar evolution som skillnaden i allelfrekvens över tiden och dessa skillnader kan användas för karakterisering av populationer. I denna studie undersöktes den genetiska variabiliteten mellan inhemska nötraser i Indonesien. För detta syfte användes DNA från 160 boskap från Aceh provinsen, 10 Bali boskap, två Madura boskap, 2 Pesisir boskap samt 2 Ongole boskap. För analys av arternas ursprung användes 20 mikrosatelliter som är genetiska markörer och för att spåra maternellt ursprung analyserades mtDNA, D-loop. Mikrosatelliterna var polymorfa i alla raser med 2-12 funna alleler med undantag för Banteng där HEL 13 och INRA35 var monomorfa. Medel heterozygositeten beräknade över 16 loci för varje ras varierade mellan 0, 3924 (Banteng) och 0, 7860 (Ongole). Inavelskoefficienten F_{IS} vilket indikerar genetisk variation inom en ras varierade mellan 0, 08851 (Pesisir) och 0, 14251 (Madura). Ett fylogenetiskt träd sk. Neighbor Joining träd visade att det genetiska släktskapet bland mtDNA sekvenserna bildade tre grupper, *Bos taurus, Bos indicus, Bos banteng* beroende på vilket ursprung individerna har. I ett median joining network bildade individer med *Bos indicus* ursprung två stjärnformade grupper. Dessa grupper är åtskilda av 4 mutationer och en haplogrupp specifik för Aceh boskapen. Sammanfattningsvis så är Banteng den mesta hotade rasen med lägst genetisk variation i jämfört med de andra raserna i studien. Aceh boskapen visade högst nivå av genetisk variation och är förmodligen en ras som använt en stor del av populationen som avelsdjur. I median joining network avslöjades en haplogrupp unik för Aceh boskapen och med den en möjligt oberoende domesticeringsprocess för zebuboskap i Indonesien.

Introduction

Personal involvement

In my master thesis I studied protein-protein interactions involved in cancer development and my interest for research and development was deepened during that time. My studies at SLU have also given me knowledge in subjects that I am really interested in and given me the ambition to continue working within this field. As I had succeeded in achieving enough study credits to obtain my Master of Science degree in a shorter time than calculated, I decided to spend one semester to pursue a second Masters project to obtain more research experience in molecular biology/genetics. In my last scheduled course, spring 2007 I made contact with Göran Andersson and asked if he had any projects that could be of interest and which I could participate in. He introduced me to the Indonesian cattle project and that is how I got involved.

General picture

Are cattle in Indonesia genetically different from cattle in Europe and what could the nature of that difference be, and are they developed through a separate evolutionary process? In recent years the developments of tools to genetically determine differences within and between species have drastically increased. This may lead to a better understanding in how evolutionary processes occur. This paper investigates the genetic variability within specific indigenous Indonesian cattle breeds.

Diversity

Biodiversity found on Earth today is the result of 3,5 billion years of evolution and the definition of biodiversity depends on what is fundamental for the user. A definition used is the "totality of genes, species and ecosystems of a region" (Spash *et al* 1995). This phrase expresses the essential circumstances and presents the traditional three levels at which biodiversity is defined and also identified:

- Genetic diversity – diversity at genetic loci within a species. There is a genetic variability among the populations and the individuals of the same species.
- Species diversity – diversity between species in an ecosystem.
- Ecosystem diversity – diversity at a higher level of organization, the ecosystem.

There are many reasons why it is important to investigate the genetic diversity of different cattle breeds and there have been several extensive studies performed around the world within and between different breeds not only in cattle but also in other domesticated farm animals such as chickens (Kerje *et al* 2003), sheep and pigs (Larson *et al* 2007).

Prior to conservation of a certain breed it is important and necessary to understand the genetic relationship and genetic variation among and between different cattle breeds to be able to preserve economical characteristics such as pathogen susceptibility, the ability to yield good quality meat (Verkaar *et al* 2002), an adaptation to poor quality fodder and to the local environment. To divide cattle into separate breeds could also, in spite of

phenotypic similarities, be accepted due to geographical, cultural and historical reasons (Hall 2004).

It is possible to investigate the origin of domestic livestock by using mitochondrial DNA diversity and further reconstruct phylogenetic relationships of sequences sampled from different geographical regions. This could, together with aurochs specimens, give us a better understanding about the history of cattle as there is a lack of wild cattle populations that could help reveal history of domestication processes and possible human interactions.

Furthermore, to loose the diversity in cattle breeds due to human actions can give indirect consequences that are unpredictable and it is therefore important to conserve genetic variation because of the unawareness of the consequences of losing that diversity. It is conceivable that the genetic diversity we have today among our domestic species is beneficial and needed to be able to respond to future challenges. These challenges could be emerging diseases, changes in environment due to climate changes and shortage of grazing land.

Cattle domestication

Livestock domestication is a complex process (reviewed by Bruford *et al* 2003) and most domesticated species are the result of multiple domestication events. Today there are various cattle types such as Yaks, Gaur, and Bali but almost all of the domesticated cattle breeds worldwide are of either *Bos taurus*, humpless type or *Bos indicus,* humped type. Supposedly, they originate from a common ancestor that diverged for more than two hundred thousand years ago which led to two separate domestication events (Loftus *et al* 1994).

The domestication of *Bos taurus* took place in the Near East about 10,000 years ago (Edwards *et al* 2007), while *Bos indicus* has it origins in the Indian subcontinent (Neolithic societies of Baluchistan in present-day Pakistan) 8,000 years ago (MacHugh *et al* 1997). The two wild progenitor populations (*Bos primigenius nomadicus, Bos primigenius primigenius)* are now extinct and died out in most regions 2,000 years ago (Bradley *et al* 1996).

The Yaks (*Bos grunniens)* present in and around the Tibet (Nguyen *et al* 2005) and Banteng (*Bos javanicus/banteng)* present in Indonesia can interbreed with *Bos taurus* and *Bos indicus*.

In more extensive studies and genetic investigations, Troy *et al* 2001 identified a four star-like cluster when examining the mtDNA control region sequences from 392 modern *Bos taurus*. The clusters showed clearly geographic differentiation where African diversity is composed of a separate haplogroup denoted T1. The haplogroup T3 predominates in Western Europe and in Near East T, T2 and T3 are present. A fourth cluster, T4 was observed in cattle from Mongolia (Mannen *et al* 2004). Phylogenetically different sequences with clearly geographic differentiation could be a sign of an independent domestication of different modern cattle breeds.

In a 2005 study, Baig *et al* constructed a reduced median network from *Bos indicus* mtDNA control region sequences. These mtDNA sequences are from the same region as

the above-mentioned sequences from *Bos taurus* and the results are therefore comparable. The results showed that the *Bos indicus* sequences form two star-like clusters, designated as Z1 and Z2. As the analysis of the mtDNA control region for *Bos indicus* is not investigated to the same extent as for *Bos taurus,* it is not conclusively established whether or not there have been independent domestication events of the wild ancestor in separate areas forming the two clusters, Z1 and Z2 but there are supportive evidence pointing in that direction.

Cattle in Indonesia

In Indonesia there about 13,5 million cattle and buffalo (2004) and they are mainly used for milk and meat production, pulling power and as fertilizer (www.fao.org). There are mostly small holder farming systems which heavily depend on natural feed resources and the survival traits for the genotypes are extremely important in the harsh low input/ low output management systems (www.aciar.gov.au). Most of the cattle originate from imported zebu and about one third is Bali cattle.

There are four existing indigenous cattle breeds in Indonesia. The Aceh cattle, which is spread all over the Aceh province and has a *Bos indicus* origin, is hold as a resource of national meat and is also used for transportation. Their ability to adapt to local conditions such as restricted food and water access is favored (Abdullah *et al* 2007). The Bali cattle has been a subject for many studies (Nijman *et al* 2003) and has a *Bos banteng* origin and is the domestic descendant of the wild Banteng. They are kept throughout Indonesia and Malaysia and their small posture makes them ideally suited for work on small- and irregular-shaped fields.

Cross breeding with Zebu cattle has occurred and the introgression of *Bos indicus* in Bali cattle varies depending on region. On the Indonesian island of Madura the Madura cattle is dominant. Traditionally it is considered to be a cross between *Bos banteng* and *Bos indicus* but it can also be a cross between *Bos taurus* and *Bos indicus* (Popescu *et al* 1988). Advantages of the Madura cattle are; high heat tolerance, feed efficiency, high quality carcass and resistance to parasites. Finally, on the coastal area of West Sumatra the Pesisir cattle are present and unfortunately relatively little is known about this breed (Djajanegara *et al* 1995). An unpublished result by K. Mohammad at the Bogor Agricultural University, Indonesia revealed *Bos indicus* origin for Pesisir cattle (Table 13).

Microsatellites

Genetic characterization of animals at the molecular level can be performed in different ways. Microsatellite markers are widely used for such purposes because they are easy to analyze, highly polymorphic and almost evenly distributed throughout the genome. Microsatellites are regions of di, tri or tetra nucleotides that can be repeated up to 100 times. The differences in repetitions give rise to allelic variation, *i.e*. genetic polymorphism. As there often are many alleles present at a microsatellite locus they can be used to estimate the divergence between and within different breeds. The analysis of genetic diversity using microsatellites provides information about the allele frequency differences among populations and it is also possible to reveal the cladistic relationship between alleles and groups of alleles by comparing the differences in allelic repeat length (MacHugh *et al* 1997). It is possible to obtain sufficient amount of amplified segment (Griffiths *et al* 2005)

to detect different alleles at marker loci when run on a gel or in a capillary instrument, by using the polymerase chain reaction (PCR) and primers flanking the repeats.

The genetic diversity can be expressed as levels of heterozygosity and in population genetics it is commonly referred to the population as a whole, *i.e*. the fraction of individuals in a population that are heterozygous for a particular locus. As only a part of the existing animals in a breed contributes to the gene pool a comparison between observed heterozygosity (H_O) in a subpopulation (breed) and the expected heterozygosity (H_E) in the whole population, will result in a decreased value of observed heterozygosity (H_O).

In 1969 Wright introduced a fixation index (F) that measures the reduction of heterozygosity at three levels: individual (I), subpopulations (S) and total population (T). F_{IS} is the inbreeding coefficient of individual (I) relative to the subpopulation (S) and F_{IS} is the estimation of H_O in relation to H_E of the subpopulations it belongs to. F_{ST} is the fixation index of the subpopulation (S) relative to the total population (T), and F_{ST} is the estimation of H_O in the subpopulation in relation to H_E of the total population. A value of one represents total differentiation and zero represents no differentiation at all between breeds.

Mitochondrial DNA (mtDNA) D-loop

The mitochondria and mitochondrial genes are in most cases and in all animals including human, inherited through the female lineage, known as maternal inheritance. A nearly non-existing genetic recombination in mtDNA makes it a helpful tool in population genetics and evolutionary biology. The relationships between mtDNA, as they are inherited as single units from different individuals (different haplotypes) can be aligned and the nucleotide sequences for the haplotypes can be compared. The analysis of the arisen patterns can be expressed in phylogenetic trees and these trees can be helpful in understanding the probable evolutionary history of populations.

The displacement loop (D-loop) region also known as the control region where replication begins is important for phyleogeographic studies. The D-loop region is the most variable mtDNA region and because the D-loop region does not code for any genes, it is free to vary with only a few exceptions. The mutation rate in the D-loop region is among the fastest compared to the mutation rate in either the nuclear or elsewhere in mitochondrial genomes in animals. Mutations in the D-loop can effectively trace recent and rapid evolutionary changes such as within species and among very closely related species.

Aims of the present study/questions

If the hypothesis; that Aceh cattle is a separate unique breed is true, then we need to answer the questions below. In this study a unique breed intend to be a breed within a clearly defined geographical area.

- The amount of heterozygosity for a gene is a measure of genetic variation in a population. How is it with the Aceh breed?

- Is the Aceh breed well separated from the other indigenous breeds in Indonesia?

- What types of cattle have contributed to the Aceh breed, and from where do they originate?

- Could that reveal anything about the gene flow and the history of the Aceh breed?

- Is there an independent domestication process for modern zebu cattle in Indonesia?

Genomic DNA derived from the Indonesian cattle used in this study was obtained from Bogor Agricultural University. However, the undiluted DNA for every sample was supplied in single labeled tubes placed in a certain order in two racks and the positions of the last 16 single tubes in rack two did not correspond to the written documentation. An uncertainty arose whether the positions, for the last 16 samples of the diluted DNA, in the 96-well plates then were correct as they normally have the same positions as the undiluted DNA. At the end of the project when all the analysis was performed it was possible to clarify the uncertainty.

In order to answer the questions above and draw correct conclusions the possible discrepancy needed to be sorted out.

For this purpose DNA from160 cattle from the Aceh province, 10 Bali cattle, two Madura cattle, two Pesisir cattle and two Ongole descendent were collected and well documented with photos by Agus Nashri Abdullah at the Bogor Agricultural University. First, 20 standardized markers recommended by the International society for animal genetics ISAG/ FAO were used in this study for the analysis of the species origin of individual microsatellite markers in the genome and secondly, tracing of maternal lineage and history of the herd was performed by analysis of the mtDNA, D-loop.

Materials and Methods

Samples

In this study DNA, 160 modern cattle originating in the Aceh province were used and collected from four different regions (Fig. 1) and well documented with photos. 10 samples of Bali cattle provided by Government of Indonesia, two samples of Madura cattle from Madura Island, two samples of PO cattle from West Java provided by Bogor Agricultural University and two samples of Pesisir cattle from West Sumatera were also included in the study (Tables 1-5).

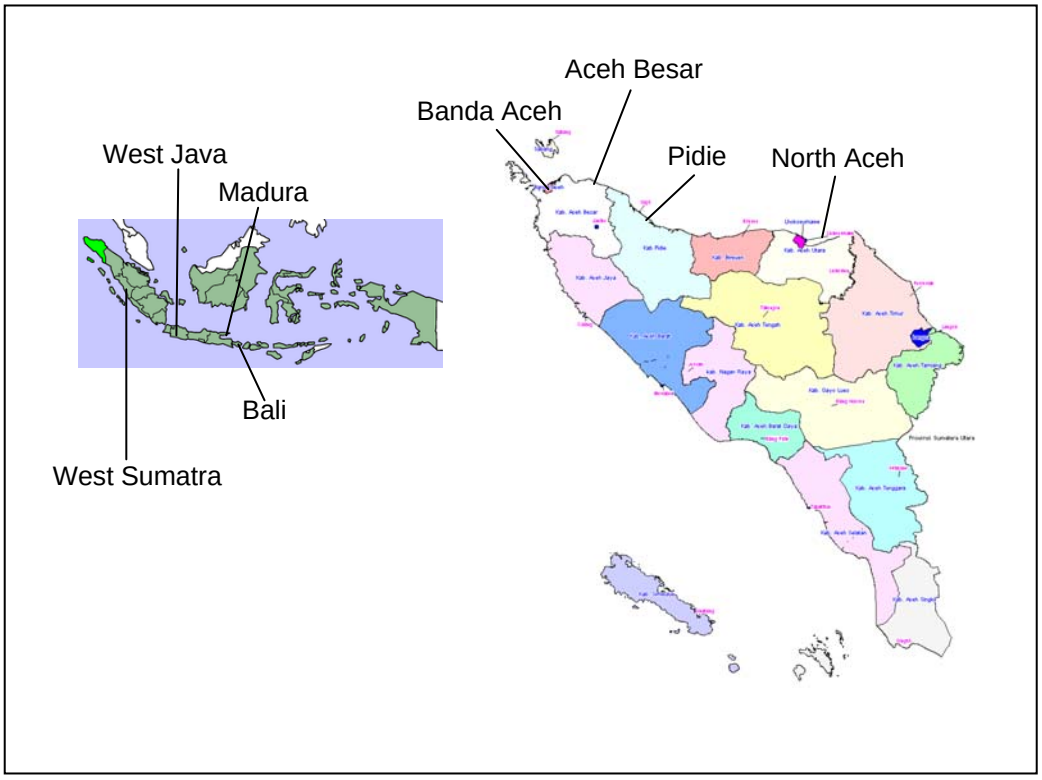

Figure 1. Map over geographical locations of cattle used in the study

Table 1. Location of Aceh cattle; AB = Aceh Besar,
SM/IJ/DI/IR = District of Suka Makmur, Ingin Jaya dan Darul Imarah, IR = Indrapuri

Sample No.	Region	Sample No.	Region
1	AB/SM	21	AB/DI
2	AB/SM	22	AB/DI
3	AB/SM	23	AB/DI
4	AB/SM	24	AB/I/IR
5	AB/SM	25	AB/I/IR
6	AB/SM	26	AB/I/IR
7	AB/IJ	27	AB/I/IR
8	AB/IJ	28	AB/I/IR
9	AB/IJ	29	AB/I/IR
10	AB/DI	30	AB/I/IR
11	AB/DI	31	AB/I/IR
12	AB/DI	32	AB/I/IR
13	AB/DI	33	AB/I/IR
14	AB/DI	34	AB/I/IR
15	AB/DI	35	AB/I/IR
16	AB/DI	36	AB/I/IR
17	AB/DI	37	AB/I/IR
18	AB/DI	38	AB/I/IR
19	AB/DI	39	AB/I/IR
20	AB/DI	40	AB/I/IR

Table 2. Location of Aceh cattle; BA = Banda Aceh,
BR/LB/UK/SK = District of Banda Raya, Lueng Bata, Ulee Kareeng, dan Syiah Kuala

Sample No.	Region	Sample No.	Region
41	BA/BR	61	BA/LB
42	BA/BR	62	BA/LB
43	BA/BR	63	BA/LB
44	BA/BR	64	BA/UK
45	BA/BR	65	BA/UK
46	BA/BR	66	BA/UK

47	BA/BR	67	BA/UK
48	BA/BR	68	BA/UK
49	BA/BR	69	BA/UK
50	BA/LB	70	BA/UK
51	BA/LB	71	BA/UK
52	BA/LB	72	BA/UK
53	BA/LB	73	BA/UK
54	BA/LB	74	BA/UK
55	BA/LB	75	BA/SK
56	BA/LB	76	BA/SK
57	BA/LB	77	BA/SK
58	BA/LB	78	BA/SK
59	BA/LB	79	BA/SK
60	BA/LB	80	BA/SK

Table 3. Location of Aceh cattle; P = Pidie,
PT/GB/UG = District of Padang Tidji, Glumpang Baro dan Ulee Glee

Sample No.	Region	Sample No.	Region
81	P/PT	101	P/GB
82	P/PT	102	P/GB
83	P/PT	103	P/GB
84	P/PT	104	P/GB
85	P/PT	105	P/GB
86	P/PT	106	P/GB
87	P/PT	107	P/GB
88	P/PT	108	P/GB
89	P/PT	109	P/GB
90	P/PT	110	P/GB
91	P/PT	111	P/GB
92	P/PT	112	P/GB
93	P/GB	113	P/GB
94	P/GB	114	P/GB
95	P/PT	115	P/GB
96	P/PT	116	P/UG
97	P/PT	117	P/UG
92	P/PT	118	P/UG
99	P/PT	119	P/UG
100	P/PT	120	P/UG

Table 4. Location of Aceh cattle; AU = Aceh Utara,
BB/MB/LS/CG = District of Baktiya Barat, Muara Batu, Lhok Seukon dan Cot Girek

Sample No.	Region	Sample No.	Region
121	AU/BB	141	AU/LS
122	AU/BB	142	AU/LS
123	AU/BB	143	AU/LS
124	AU/BB	144	AU/LS
125	AU/BB	145	AU/CG
126	AU/BB	146	AU/CG
127	AU/BB	147	AU/CG
128	AU/BB	148	AU/CG
129	AU/BB	149	AU/CG
130	AU/BB	150	AU/CG
131	AU/BB	151	AU/CG
132	AU/MB	152	AU/CG
133	AU/MB	153	AU/CG
134	AU/MB	154	AU/CG

135	AU/BB	155	AU/CG
136	AU/BB	156	AU/CG
137	AU/BB	157	AU/CG
138	AU/BB	158	AU/CG
139	AU/LS	159	AU/CG
140	AU/LS	160	AU/CG

Table 5. Location of Bali cattle (B), Ongole descendant (PO), Madura (M), Pesisir (PS)

Sample No.	Region	Sample No.	Region
B1	Bali Island	B10	Bali Island
B2	Bali Island	PO1	West Java
B3	Bali Island	PO2	West Java
B4	Bali Island	M1	Sumenep, East Java
B5	Bali Island	M2	Sumenep, East Java
B6	Bali Island	PS1	West Sumatera
B7	Bali Island	PS2	West Sumatera
B8	Bali Island		
B9	Bali Island		

Microsatellite analysis

Microsatellite markers according to Table 6 were chosen from the panel of 30 standardized markers recommended by the International society for animal genetics ISAG/ FAO to screen the breeds for diversity. For two of the markers, INRA023 and INRA032 some optimization of the PCR was performed but due to problems with amplification they were removed from further analysis. Primer sequences, alternative name and size range are shown in Table 6.

Table 6. 20 microsatellite markers recommended by FAO

Locus	Alternative name	Primer sequence	Size range (bp)
HEL1	D15S10	F:CAACAGCTATTTAACAAGGA R:AGGCTACAGTCCATGGGATT	99-119
HEL13	D11S15	F:TAAGGACTTGAGATAAGGAG R:CCATCTACCTCCATCTTAAC	178-200
ETH3	D19S	F:GAACCTGCCTCTCCTGCATTGG R:ACTCTGCCTGTGGCCAAGTAGG	103-133
ETH10	D5S3	F:GTTCAGGACTGGCCCTGCTAACA R:CCTCCAGCCCACTTTCTCTTCTC	207-231
ETH152	D5S1	F:TACTCGTAGGGCAGGCTGCCTG R:GAGACCTCAGGGTTGGTGATCAG	181-211
ETH255	D9S1	F: GATCACCTTGCCACTATTTCCT R:ACATGACAGCCAGCTGCTACT	131-159
INRA005	D12S4	F:CAATCTGCATGAAGTATAAATAT R:CTTCAGGCATACCCTACACC	135-149
INRA035	D16S11	F:ATCCTTTGCAGCCTCCACATTG R:TTGTGCTTTATGACACTATCCG	100-124
INRA063	D18S5	F:ATTTGCACAAGCTAAATCTAACC R:AAACCACAGAAATGCTTGGAAG	167-189
BM1824	D1S34	F:GAGCAAGGTGTTTTTCCAATC R:CATTCTCCAACTGCTTCCTTG	176-197
BM2113	D2S26	F:GCTGCCTTCTACCAAATACCC R:CTTCCTGAGAGAAGCAACACC	122-156
BM1818	D23S21	F:AGCTGGGAATATAACCAAAGG R:AGTGCTTTCAAGGTCCATGC	248-278
ILST005	D10S25	F:GGAAGCAATGAAATCTATAGCC	176-194

		R:TGTTCTGTGAGTTTGTAAGC	
ILST006	D7S8	F:TGTCTGTATTTCTGCTGTGG R:ACACGGAAGCGATCTAAACG	277-309
CSRM60	D10S5	F:AAGATGTGATCCAAGAGAGAGGCA R:AGGACCAGATCGTGAAAGGCATAG	79-115
CSSM66	D14S31	F:ACACAAATCCTTTCTGCCAGCTGA R:AATTTAATGCACTGAGGAGCTTGG	171-209
HEL5	D21S15	F:GCAGGATCACTTGTTAGGGA R:AGACGTTAGTGTACATTAAC	145-171
HEL9	D8S4	F:CCCATTCAGTCTTCAGAGGT R:CACATCCATGTTCTCACCAC	141-173
INRA023	D3S10	F:GAGTAGAGCTACAAGATAAACTTC R:TAACTACAGGGTGTTAGATGAACTCA	195-225
INRA032	D11S9	F:AAACTGTATTCTCTAATAGCTAC R:GCAAGACATATCTCCATTCCTTT	160-204

Amplification of the individual loci was performed by PCR with the same reaction conditions for all markers. Reactions were performed using 96-well microtitre plates. The PCR was run in GeneAmp® PCR System 9700 with a touchdown program. The master mix had a total volume of 10µl and contained following reagents:

10X AmpliTaq Gold® buffer without $MgCl_2$	1 µl
$MgCl_2$	1 µl
dNTP (2mM)	2 µl
Primer F (20 µM)	0,1 µl
Primer R (20 µM)	0,5 µl
M13-oligo (20 µM)	0,5 µl
DNA (5 ng/ µl)	2 µl
AmpliTaq Gold® DNA polymerase	0,2 µl
ddH_2O	2,7 µl

Cycling conditions according to following program:

95° C	95° C	65-52° C	72° C	95° C	52° C	72° C	72° C	4° C
10 min	30 sec	30 sec	30 sec	30 sec	30 sec	30 sec	7 min	∞
	14 cycles decreasing 1° C each cycle			30 cycles				

Prior to the analysis a mix containing 1200µl Hi-Di Formamide and 25µl GeneScan™-LIZ 500 Size Standard was prepared and 14µl of this was added to 1µl PCR product. After heat treatment at 95° C for 3 minutes the PCR products were separated by capillary electrophoresis using ABI PRISM™ 3100 Genetic Analyzer and the data was collected using the 3100 Data Collection Software Version 2,0. The alleles were assigned using Genemapper™ Software Version 3,7. A reference sample was used to standardize the allele sizes when genotyping the samples.

Data analysis

The results obtained from the genetic analyses, general population statistics such as allele number, allele frequencies, expected and observed heterozygosity were calculated using Microsoft Toolkit. Pair-wise comparison of F_{ST} values between population pairs was

calculated using GENEPOP 3.4, F_{ST} is estimated as in Weir and Cockerham 1984. Finally the F_{IS} values were obtained by using GENETIX 4.05.

Another study was performed in Indonesia (unpublished Mohammad *et al* 2007), earlier in 2007, using the same breeds as in this study. Since supervisor Mia Olsson performed the genetic analysis for the microsatellites the opportunity to combine some of the material (Table 7) with the material in the present study was possible. These samples have been genotyped with the same set of markers besides HEL5 and HEL9 and the allele sizes had been standardized in the same manner. To be able to combine the two sets of genotyped individuals, markers HEL5 and HEL9 where removed from the individuals in this study.

Table 7. Samples provided by K Mohammad included in the microsatellite analysis

Breed	**No. of individuals**	**Region**
Banteng	9	Ragunan zoo
Bali	39	Bali Island
Madura	26	East Java Al centre, Singosari
Pesisir	27	West Sumatra
Aceh	16	Aceh Sumatra
PO Ongole	5	South Sumatra

Methods for D-loop sequencing

The D-loop region of mtDNA was amplified using PCR with three different primer pairs. Primer sequences are shown in Table 8. From the beginning, primer pair one was already designed and available in the laboratory but there had earlier been problems with the amplification of the 980 bp fragment from the individuals in the present study using this primer pair. The reason for that was not quite clear since the primer pair, PCR-protocol and cycling conditions worked when using DNA from *Bos taurus*. Therefore, the cycling conditions and protocol were adjusted and half of the individuals in the present study were successfully amplified. At that moment more knowledge revealed that primer pair one was designed for *Bos taurus* and not for *Bos indicus* which was the origin of most individuals in the present study. For that reason, two more primer pairs were used one amplifying 720 bp fragment and one amplifying 177 bp fragment. Primer pair three, amplifying the shortest fragment, was thought to be used in case of primer pair two not working.

Table 8. Primer pairs for amplification of mtDNA D-loop

Primer pair	**Name**	**Primer sequence**	**Length in bp**	**Reference**
1	BIDL f	ACCCCCAAAGCTGAAGTTCT	980	
	BIDL r	GTGCCTTGCTTTGGGTTAAG		
2	Beth Big F	ACCCCCAAAGCTGAAGTTCT	720	G.Larson
	Beth 80r	CAAGCATCCCCCAAAATAAA		G.Larson
3	Beth 178f	CCCCATGCATATAAGCAAG	177	G.Larson
	Beth 309r	GCCTAGCGGGTTGCTGGTTTCACGC		G.Larson

Protocol and cycling conditions for primer pair 1 run on all samples:

10X AmpliTaq Gold® buffer without $MgCl_2$	1 µl
$MgCl_2$	2 µl
dNTP (2mM)	0,2 µl

Primer F (20 µM)	0,5 µl
Primer R (20 µM)	0,5 µl
DNA (5 ng/ µl)	2 µl
AmpliTaq Gold® DNA polymerase	0,2 µl
ddH_2O	3,6 µl

Cycling conditions for primer pair 1:

95° C	95° C	65-58° C	72° C	95° C	58° C	72° C	72° C	4° C
5 min	30 sec	30 sec	30 sec	30 sec	30 sec	30 sec	10 min	∞
	14 cycles touchdown with 0,5° C each cycle			30 cycles				

Protocol and cycling conditions for primer pair 2 run on all samples:

10X AmpliTaq Gold® buffer without $MgCl_2$	2,5 µl
$MgCl_2$	2,5 µl
dNTP (2mM)	0,25 µl
Primer F (20 µM)	1,25 µl
Primer R (20 µM)	1,25 µl
DNA (5 ng/ µl)	1 µl
AmpliTaq Gold® DNA polymerase	0,25 µl
ddH_2O	16 µl

Cycling conditions for primer pair 2:

94° C	94° C	56° C	72° C	72° C	4° C
2 min	45 sec	45 sec	1,5min	10 min	∞
	35 cycles				

Protocol and cycling conditions for primer pair 3 run on ambiguous samples 6,13, 20, 35, 36, 41, 49, 50, 54, 60, 61, 64, 65, 66, 67, 73, 74, 75, 76, 77, 78, 80, 81, 82, 90, 91, 92, 93, 95, 98, 101, 102, 103, 104, 105, 110, 112, 113, 114, 115, 116, 118, 119, 121, 122, 129, 130, 134, 137, 138, 141, 153, 158, B1-B10, M1-M2, PS1-PS2, PO1-PO2 are the same as for primer pair 2.

To verify that the correct products were obtained in all the amplifications, the reactions were loaded on a 2% EtBr-stained agarose gel and to remove unincorporated dNTPs and primers a quick PCR clean-up was performed with ExoSAP according to the manufacture's instructions.

All the samples were prepared according to the instruction provided by Uppsala Genome Center prior to sequencing in Big Dye® Terminator v3.1. Sequencing was performed using the same primer pairs they had been amplified with except for primer pair 1 that was only sequenced with the forward primer.

Data analysis

Contigs were reconstructed from the set of overlapping DNA sequences for all individuals except for samples 30, 34, 85,107,116, B2, B3, B5, B8 and PO2 using Codon Code

Alinger. Multiple sequence alignments with the complete sequences of the mtDNA D-loop and sequences from previously published studies and sequences provided by G Larson according to Table 9 were performed using Se-Al (Rambaut 1996). For the inference of un-rooted neighbour joining (NJ) trees and determination of haplotypes the software implemented program PAUP 4.0 (Swofford 1996) was used. Finally, median joining networks were constructed by using NETWORK 4.500 (Bandelt *et al* 1999).

Table 9. Reference samples for multiple alignments retrieved from Genbank and G.Larson

Abbreviation	**Name/origin**	**Species**	**Accession no D-loop, mtDNA**	**Reference**
	T	*Bos taurus*		G.Larson
	T1	*Bos taurus*		G.Larson
	T2	*Bos taurus*		G.Larson
	T3	*Bos taurus*		G.Larson
	T4	*Bos taurus*		G.Larson
	T3 long	*Bos taurus*		G.Larson
G1	Bos Taurus brown	*Bos taurus*	AF0341038	
G2	XG-1 Taurus	*Bos taurus*		G.Larson
G3	XG-3 Taurus	*Bos taurus*		G.Larson
G4	Cuc-18 Taurus	*Bos taurus*		G.Larson
G5	57 Guini Taur	*Bos taurus*		G.Larson
G6	58 Guini Taur	*Bos taurus*		G.Larson
G7	59 Guini Taur	*Bos taurus*		G.Larson
G8	14.28 TaurIndicus	*Bos taurus*		G.Larson
G9	Indicus	*Bos indicus*	AF162485	Nijman *et al* 2003
G10	Indicus	*Bos indicus*	AF162484	Nijman *et al* 2003
G11	Zebu 2	*Bos indicus*		G.Larson
G12	Zebu 1	*Bos indicus*		G.Larson
G13	Buhtan	*Bos indicus*	AB268581	Lin *et al* 2007
G14	Buhtan	*Bos indicus*	AB268580	Lin *et al* 2007
G15	Buhtan	*Bos indicus*	AB268579	Lin *et al* 2007
G16	Buhtan	*Bos indicus*	AB268578	Lin *et al* 2007
G17	Buhtan	*Bos indicus*	AB268577	Lin *et al* 2007
G18	41Nindia	*Bos indicus*		G.Larson
G19	42Nindia	*Bos indicus*		G.Larson
G20	43Nindia	*Bos indicus*		G.Larson
G21	46Sindia	*Bos indicus*		G.Larson
G22	47Sindia	*Bos indicus*		G.Larson
G23	48Sindia	*Bos indicus*		G.Larson
G24	Banteng Bali	*Bos indicus*		G.Larson
G25	Banteng Bali	*Bos indicus*	AF162486	Nijman *et al* 2003
G26	Banteng Bali	*Bos banteng*	AF162490	Nijman *et al* 2003
G27	Banteng Bali	*Bos banteng*	AF162489	Nijman *et al* 2003
G28	Madura	*Bos banteng*	AF162488	Nijman *et al* 2003
G29	Madura	*Bos banteng*	AF162487	Nijman *et al* 2003
G30	Banteng 1	*Bos banteng*		G.Larson
G32	Banteng 2	*Bos banteng*		G.Larson
G32	Indicus Madura 2	*Bos banteng*		G.Larson
G33	Indicus Madura 1	*Bos banteng*		G.Larson
C11	BOV THAB India	*Bos indicus*	L27737	Kim *et al* 2003
C12	BOV THAA India	*Bos indicus*	L27736	Kim *et al* 2003
C13	BOV HARA India	*Bos indicus*	L27722	Kim *et al* 2003
C14	BOV HARB India	*Bos indicus*	L27723	Kim *et al* 2003
C15	BOV SAHA India	*Bos indicus*	L27733	Kim *et al* 2003
C16	BOV SAHB India	*Bos indicus*	L27732	Kim *et al* 2003

C19	China	*Bos indicus*	EF417985	Baig *et al* 2005
C20	China	*Bos indicus*	EF417974	Baig *et al* 2005
C21	China	*Bos indicus*	AB268579	Baig *et al* 2005
C22	China	*Bos indicus*	AB268556	Baig *et al* 2005
C23	China	*Bos indicus*	AY378136	Baig *et al* 2005
C24	China	*Bos indicus*	AY902403	Baig *et al* 2005
C25	China	*Bos indicus*	EF417971	Baig *et al* 2005
C26	India	*Bos indicus*	AY972130	Baig *et al* 2005
C27	India	*Bos indicus*	AY972131	Baig *et al* 2005
C28	India	*Bos indicus*	AY972132	Baig *et al* 2005
C29	India	*Bos indicus*	AY972133	Baig *et al* 2005
C30	India	*Bos indicus*	AY972134	Baig *et al* 2005
C31	India	*Bos indicus*	AY972135	Baig *et al* 2005
C32	India	*Bos indicus*	AY972136	Baig *et al* 2005
C33	India	*Bos indicus*	AY972137	Baig *et al* 2005
C34	India	*Bos indicus*	AY972138	Baig *et al* 2005
C35	India	*Bos indicus*	AY972139	Baig *et al* 2005
C36	India	*Bos indicus*	AY972140	Baig *et al* 2005
C37	India	*Bos indicus*	AY972141	Baig *et al* 2005
C38	India	*Bos indicus*	AY972142	Baig *et al* 2005
C39	India	*Bos indicus*	AY972143	Baig *et al* 2005
C40	India	*Bos indicus*	AY972144	Baig *et al* 2005
C41	India	*Bos indicus*	AY972145	Baig *et al* 2005
C42	India	*Bos indicus*	AY972146	Baig *et al* 2005
C43	India	*Bos indicus*	AY972147	Baig *et al* 2005
C44	India	*Bos indicus*	AY972148	Baig *et al* 2005
C45	India	*Bos indicus*	AY972149	Baig *et al* 2005
C46	India	*Bos indicus*	AY972150	Baig *et al* 2005
C47	India	*Bos indicus*	AY972151	Baig *et al* 2005
C48	India	*Bos indicus*	AY972152	Baig *et al* 2005
C49	India	*Bos indicus*	AY972153	Baig *et al* 2005
C50	India	*Bos indicus*	AY972154	Baig *et al* 2005

Results

Microsatellite analysis

One of the tasks was to amplify the DNA from all individuals in the different cattle breeds and to run the samples in a multi-color fluorescence-based DNA genetic analyzer. In this study 160 Aceh cattle, 10 Bali cattle, two Pesisir cattle, two Madura cattle and two Ongole descendant cattle were successfully genotyped.

In order to determine the genetic relationships between the four groups of Aceh cattle a pair-wise comparison showed that there were minor differences (Table 10) depending on sampling region and in further analysis the Aceh cattle were treated as one homogenous group.

Table 10. Pair-wise comparison of F_{ST} values between Aceh groups

Population	**Banda Aceh**	**Aceh Besar**	**Aceh Pidie**
Aceh Besar	-0,003		
Aceh Pidie	-0,001	-0,0041	
Aceh North	0,0017	0,0028	0,0034

Another task was to analyze obtained data together with the genotyped individuals provided by K. Mohammad and to calculate basic genetic information using different software implemented programs. Matching samples were removed (Az307, Az311, Az293, Az310, Bb120) The microsatellites were polymorphic in all breeds, ranging from three to twelve detected alleles with the exception for the Banteng where HEL13 and INRA35 were monomorphic and the detected alleles for the other loci ranged from two to four.

The mean number of alleles per locus (MNA) observed in each breed (Table 11) is considered to be a reasonable indicator of genetic variation assuming that the populations are in mutation-drift equilibrium and of similar sample size. The results showed that the level of observed heterozygosity (HO) was lower than the expected heterozygosity (HE) for all breeds and Table 11 shows mean heterozygosity computed across the 16 loci for each breed which ranged between 0, 3924 (Banteng) and 0, 7860 (Ongole). The inbreeding coefficient F_{IS} which indicates within breed genetic variation ranged from 0, 08851 (Pesisir) to 0, 14251 (Madura). Two of the breeds, Ongole and Banteng were excluded from the F_{IS} analysis since the number of individuals need to be minimum 10 to perform this analysis.

Table 11. Microsatellite toolkit, population statistics for each studied breed

Breed	Sample size	Unbiased Heterozygosity H_E	Observed Heterozygosity H_O	Mean # of alleles/loci MNA	# of polymorphic loci	# of private alleles	F_{IS} Value
Aceh	170	0,6753	0,6152	8,81	16	19	0,08916
Bali	49	0,6439	0,5670	6,25	16	0	0,12071
Banteng	9	0,3728	0,3924	2,19	14	0	N/A
Madura	29	0,7469	0,6421	7,63	16	2	0,14251
Ongole	7	0,7836	0,6860	5,44	16	3	N/A
Pesisir	28	0,6653	0,6075	6,00	16	2	0,08851

In order to determine the genetic relationships between the six cattle breeds used in the study, a pair-wise comparison showed that the Banteng compared to the other breeds had the highest F_{ST} values. The lowest value in the pair-wise comparison was between Ongole and Madura.

Table 12. Pair-wise comparison of F_{ST} values between cattle breeds

Population	Bali	Banteng	Ongole	Madura	Aceh
Banteng	0,2213				
Ongole	0,1776	0,3570			
Madura	0,1735	0,3002	0,0250		
Aceh	0,2315	0,3484	0,0418	0,0881	
Pesisir	0,2319	0,3590	0,0447	0,0825	0,0973

D-loop sequencing analysis

All individuals were successfully sequenced except for samples 30, 34, 85, 107, 116, PO2, B2, B3, B5 and B8. The reason for not succeeding with all samples was due to bad amplification, limited amounts of DNA and too much background in the sequences. The length of the sequences varied, and sample 60, 82, 101, 102, 115, 118, 119, 130 were amplified only with primer pair 3 and their sequences ranged between 98-230 bp.

An un-rooted Neighbour Joining (NJ) tree with all samples and aligned reference samples grouped the control region sequences into three distinct lineages; *Bos taurus, Bos indicus and Bos banteng*.

The possible discrepancy regarding positions of the 16 samples in the 96-well plate was an important problem to solve and a more thorough investigation of the NJ tree was performed. In the obtained NJ tree, individuals with the same maternal origin clustered. If the 16 individuals in the present study would have had the positions according to the real/true position of the undiluted DNA, the Pesisir and Ongole samples would have clustered with reference samples of maternal Indicine origin and the Bali samples would have clustered with reference samples of maternal Banteng origin. Other studies (Nijman *et al* 2003, Mohammad *et al,* unpublished) demonstrate that the breeds, Pesisir and Ongole mostly have maternal Indicine origin and Bali cattle from the island of Bali have Banteng origin (Table 13) which corresponds with the results from the NJ tree analysis in this study. Therefore, it is most likely that the real/true position of the undiluted DNA, of the 16 samples, corresponds to the positions in the 96-well plate.

Table 13. A comparison of maternal origin for different cattle breeds. The compared breeds, where the samples are collected, total numbers of each breed in the studies and number of banteng/indicine maternal origin respectively are highlighted.

Breed	**Region**	**Tot. no of animals in the study**	**Maternal origin**		**Reference**
			Banteng	**Indicine**	
Aceh	Banda Aceh	38		38	Present study
Aceh	Aceh Besar	40		40	Present study
Aceh	Pidie	36		36	Present study
Aceh	North Aceh	40		40	Present study
Madura	Madura Island	2	2		Present study
Pesisir	West Sumatra	2		2	Present study
Ongole	East Java	1		1	Present study
Bali	Bali Island	6	6		Present study
Bali	Bali Island	57	57		Lenstra unpublished
Aceh	Aceh	12		12	Lenstra unpublished
Pesisir		24		24	Lenstra unpublished
Ongole		5	2	3	Lenstra unpublished
Galekan		48	6	42	Lenstra unpublished
Madura		42	21	21	Lenstra unpublished
Bali	South Sumatra	38	38		Lenstra unpublished
Bali	Sumatra	No result			Lenstra unpublished
Bali	Sulawesi	No result			Lenstra unpublished
Madura	Slaugter house	7	4	3	Nijman *et al* 2003
Bali	Malysia	17	6	11	Nijman *et al* 2003
Bali	Bali Island	11	11		Nijman *et al* 2003
Banteng	Zoo	4	4		Nijman *et al* 2003

Within the control region sequences examined, 53 haplotypes were defined for the 155 assayed Aceh cattle, including one that was represented 48 times. Another haplotype was shared between 34 animals and a third haplotype was shared seven times. Four other haplotypes were found in triplicate and nine in duplicate and rest of the haplotypes as single samples.

The phylogenetic relationship observed in the NJ tree was further demonstrated by constructing a median joining network (Fig. 2) from the 230 bp mtDNA control region for

the Aceh cattle, two Pesisr cattle, one Ongole descendant and the reference samples in order to focus on the relationship between the Ache breed and other zebu cattle from Africa, China, Buthan and India. To be able to create a Phylip file, which is necessary for construction of the median joining network, from the NJ tree all degenerate codes had to be translated to the most likely nucleotides (Table 14). The 230 bp region is known to be the most variable fragment of the mtDNA control region and have been used in other studies (Nijman *et al* 2003, Baig *et al* 2005, Kim *et al* 2003, Loftus *et al* 1994).

The sequences grouped into two star-like clusters with predominant haplotypes in the center with 78 respectively 14 sequences (Fig. 1). The two clusters are separated by four mutations and one haplotype unique for 39 Aceh cattle. In total, 47 haplotypes were revealed from 204 sequences and all mutations were transitions except for two that were transversions. Zebu cattle from all regions were present in both of the star-like clusters and no significant regional difference could be observed between the different regions from where the Aceh cattle were sampled. A network using 400 bp sequences were also constructed (Fig. 3) and showed similar pattern but with only 40 haplotypes included formed by 177 sequences. The frequency with which each haplotype occurs is indicated by the area of the circle indicating it.

Table 14. Translation of degenerate codes to the most likely nucleotides in certain samples

Sample No	Position	Degenerate code	Nucleotide
A111	179	S	G
	178	M	A
	519	M	A
A127	269	R	A
A24	336	Y	T
	521	Y	C
A14	336	Y	T
	418	Y	C
A21	337	Y	C
	362	Y	C
	373	Y	T
	635	R	A
A53	418	Y	C
A9	179	R	G
	226	R	G
	517	R	G
	521	Y	T
A93	377	Y	T
	378	Y	T
	429	Y	T
A96	377	Y	T
	378	Y	T
	429	Y	T
	521	Y	T
	521	Y	T
	521	Y	T

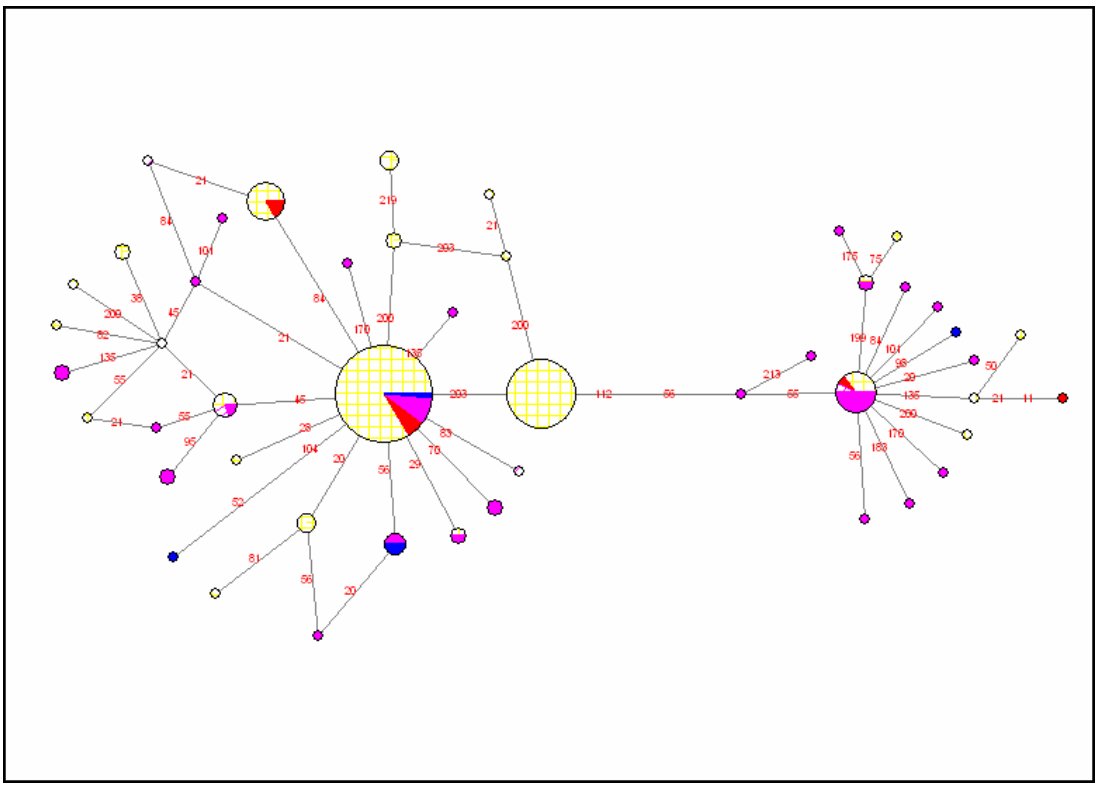

Figure2. A median joining network constructed from the 230 bp mtDNA control region of Aceh cattle (yellow cross), other Zebu cattle from Indonesia (blue) and other Zebu cattle from China (red), India (pink) and Buthan (pink striped).

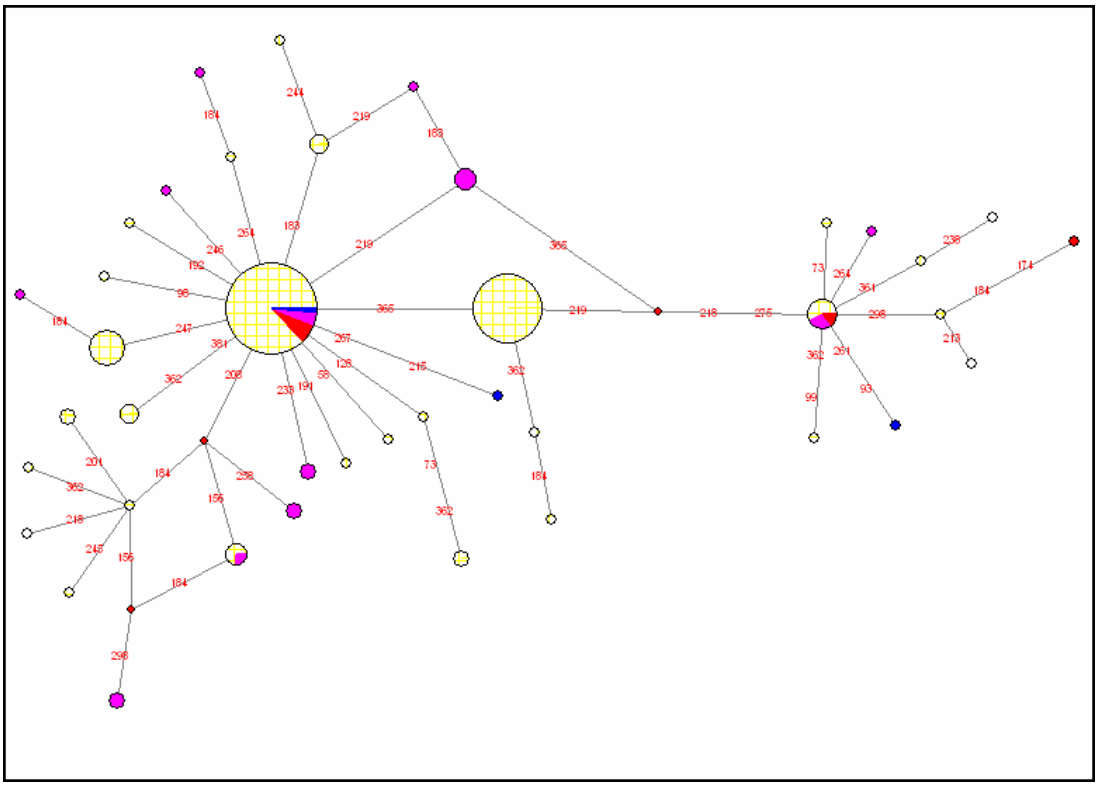

Figure 3. A median joining network constructed from the 400 bp mtDNA control region of Aceh cattle (yellow cross), other Zebu cattle from Indonesia (blue) and other Zebu cattle from China (red), India (pink) and Buthan (pink striped). Three small red circles represent hypothetical sequences not found.

Discussion

In this project, cattle from Indonesia were investigated using microsatellite- and mtDNA sequencing analysis to reveal the genetic variation within and among populations.
It is important to preserve species and also breeds for their uniqueness and when it comes to domesticated cattle mostly for their economical values but also for other reasons. In Indonesia there are several breeds with unique features such as: ability to produce milk and meat even though it is shortcoming of nutritional feed, heat tolerance and resistance to certain parasites. They are also well-suited for small holder farming systems which is most common in Indonesia. To reveal the history of the different species is also very interesting as it tells us about domestication processes and a lot of our own history.

During the time of this project minor problems have come into existence and three of them will be mentioned here. The first problem did appear already the first day when going through the documentation and DNA samples. Because all DNA is diluted from single tubes into 96-well plates and the DNA used for PCR amplification is taken from the 96-well plates it is really important to know what is what when analyzing the results. Finally, when constructing a phylogenetic tree it was possible to reveal which samples was which and this was of course very good to know, but it also resulted in that the whole microsatellite analysis had to be redone. The second problem appeared when the first part, the microsatellite analysis, was finished and it was time to start with the sequencing and analysis of the mtDNA. Initially it was not unambiguously clear how to further analyze the sequences obtained and what tools to be used. The second problem was later solved when Greger Larson became involved in the project. Finally, the third problem is unsolved and deals with extraction of more DNA from blood samples. The blood is frozen and preserved in ethanol and efforts using both salt precipitation and a Qiagen kit have been unable to yield any new DNA. If further analysis is going to take place, that problem needs to be solved as some of the individuals had run out of DNA.

Level of heterozygosity, inbreeding and differentiation

The analysis of the microsatellites was performed together with a second set of samples. One of the benefits with these samples was that the laboratory work and the calibration of alleles, with the same standard were performed by Mia Olsson who also was one of the supervisors. As expected the level of observed heterozygosity and average number of alleles turned out to be the lowest for the Banteng compared to the other breeds. The samples from Banteng are limited and collected at Ragunan Zoo and to get better and more reliable results it would be advantageous to achieve DNA from modern wild Bantengs. To compare such individuals with the individuals in the study would give more information about the genetic diversity and how inbred they are. The observed heterozygosity was highest for the Ongole and a possible explanation could be that the samples are collected from both east Java and south Sumatra resulting in a higher value despite few samples. The Aceh breed studied showed a considerable amount of within-breed variation based on allele numbers and heterozygosity values, showed the highest mean number of alleles /loci and had 19 private alleles.

The F_{IS} values for Bali and Madura cattle suggest that the represented samples are inbred but this could also be due to small sample size and/or that some of the samples considered

as unrelated may indeed share common parentage in the history beyond known pedigree. A relatively high F_{IS} value could also be a result of sex-biased dispersal, a feature of population decline or a result of the breeding system. Most likely immigration could still maintain the populations but for the Bali breed some studies (Nijman *et al* 2003; Verkaar *et al* 2003) have reported problems with introgression of Zebu despite a ban on import of other breeds to the isle of Bali, to prevent the Bali breed from getting contaminated. A study performed by R. Noor 2001 also determined that four out of eight Bali bulls at an artificial breeding center were not pure bred and that they have contaminated the purity of Bali cattle. Taken those aspects into consideration, the limited number of pure bred Bali cattle may have contributed to this low level of within genetic variation. In contradiction to Nijman *et al* 2003 who found at most microsatellite loci characteristic alleles for Bali cattle and Banteng, the use of other microsatellite markers in this study only revealed one characteristic allele for Bali and Banteng in microsatellite INRA063. The microsatellites in this study are therefore most likely not suitable for assaying the purity of Bali and other Banteng breeds.

The pair-wise comparison between the breeds in this study determined the genetic relationships to be highest between the Banteng and all the other breeds with a value ranging from 0.22-0.36. This suggests that the Banteng is well separated from the other breeds and for Aceh, Pesisir and Ongole this is not revealing becuase they have another origin, *Bos indicus* instead of *Bos banteng.* More surprisingly is it that the Bali cattle are so well separated from the Banteng. In reality, the origin should be the same for Bali and Banteng because the Bali cattle is the domestic descendant of Banteng, but the difference could also possibly be a consequence of crossbreeding as mentioned above.

The genetic distance between Aceh, Pesisir and Ongole ranged from 0.02-0.1. If the values are compared with the within breed variation for the Aceh cattle (Table 12) where it is almost no variation, these values between breeds are higher and therefore indicate some differences.

D-loop polymorphism

To further investigate the samples and reveal more hidden treasures, sequencing of the control region of the mtDNA was performed. There were huge problems amplifying the mtDNA region with primer pair 1 and only half of the samples worked. After a closer examination of the aligned references sequences it was quite clear that the reverse primer in primer pair 1 was not well designed. When eliminating that problem, new primers were designed or if it should be correct, Greger Larson already had two primer pairs designed so it was only to order them. The PCR reactions were performed and the amplified products were sent to Rudbeck Genome Center for nucleotide sequencing. Some amplifications went very well but some were more challenging and it took almost one week to make contigs of all test sequences, short sequences and long sequences and align them to the reference samples.

In the NJ tree, genetic relationships among the mtDNA sequences revealed that all samples with different origin clustered together, *Bos taurus, Bos indicus,* and *Bos banteng.* This was in accordance with other studies performed with the same reference sequences. From the NJ tree it was possible to define 53 haplogroups for the Aceh cattle where almost 53% were in two of the haplogroups.

Other studies have revealed that 240 bp of the mtDNA control region from *Bos indicus*, group into two star-like clusters and is separated by four mutations (Baig *et al* 2005). In this study the zebu sequences also grouped into two clusters and the potential center haplotypes are separated by four mutations and a unique haplotype for the Aceh cattle. In another study performed by Lai *et al* 2006, the two clusters were separated by five mutations and as sequences of all zebu cattle are not investigated, it is not certain how many mutations that should separate the two clusters. Therefore, it is likely that one of the mutations in the present study belongs to a new haplotype. Zebu cattle from all regions were present in both of the star-like clusters and no significant regional difference could be seen between the different regions from where the Aceh cattle were sampled.

If mtDNA is used as the sole source of data when investigating phyleogeographic processes there are potential risks of misinterpretations. Important sex-related demographic patterns may be overlooked unless a broad approach, encompassing mtDNA, Y-chromosome polymorphism and autosomal variation is used (MacHugh, 1997).

Conclusions

The conclusion from the microsatellite analysis is that the Banteng is the most endangered breed with low genetic variation with only 14 polymorphic loci and fewer alleles for the other loci when compared with the other breeds in the study. However, the low genetic diversity among the Banteng used in this study may be explained by their close family relationship as all were derived from a zoological garden and related. Additional Banteng samples are required to conclusively define the genetic diversity among Banteng.
On the other hand, the Aceh cattle showed a much higher level of genetic variation where all the loci were polymorphic and the detected alleles ranged from five to twelve where 19 of them were unique. The case for the Aceh breed is that it is probably a mixed breed, or rather a breed that has used a large part of the population as breeding animals which reduces the loss of alleles from the population.

One of the five mutations separating the star-like clusters in the median joining network possibly belongs to a new haplogroup so far unique for the Ache breed. It is also possible to draw the same conclusions as for the microsatellite analysis that large parts of the zebu population have been used as breeding animals and also that zebu cattle from different regions/countries have influenced the genetic variation.

Possible manuscripts and further progress

1. Journal: Nature
   Idea: Does the Aceh zebu breed have unique haplotype?
   Further progress: Look through everything and see if it is okay

2. Journal: Genetics
   Idea: Microsatellite DNA variation of Indonesian cattle
   Further progress: More computer analysis such as deviations from Hardy-Weinberg equilibrium, assignment tests, genetic distance trees

3. Journal: Animal Genetics
   Idea: Low genetic variation within Banteng. Is it endangered?
   Further progress: Find more samples and include them in the study

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