Introduction

Several bovine species have contributed worldwide to cattle livestock [1]. Most domestic cattle belong to the species Bos taurus or Bos indicus (zebu), which both descend from the wild aurochs Bos primigenius. Domestic yak (Bos grunniens) is kept in and around Tibet, the gayal (Bos frontalis) of Eastern India is derived from the gaur (Bos gaurus), while the Indonesian Bali cattle is a domestic form of the banteng (Bos javanicus). Despite their obvious role as livestock during our cultural development, the history of domestic cattle has been poorly documented. For the past 15 years, DNA analysis has allowed a phylogenetic reconstruction of the earliest events during domestication [2,3]. For instance, analysis of mitochondrial DNA established a taurine maternal origin of zebu events during domestication [2,3]. For instance, analysis of mitochondrial DNA established a taurine maternal origin of zebu introgression (20–30%) and combine a zebu paternal lineage with a predominant (Madura) or even complete (Galekan) maternal banteng origin. Two Madura bulls carried taurine Y-chromosomal haplotypes, presumably of French Limousin origin. In contrast, we did not find evidence for zebu introgression in five populations of the Bali cattle, a domestic form of the banteng.

Conclusions: Because of their unique species composition Indonesian cattle represent a valuable genetic resource, which potentially may also be exploited in other tropical regions.

Materials and Methods

Samples and DNA isolation

All animals were handled by veterinarians from the Faculty of Veterinary Medicine, Bogor Agricultural University in strict accordance with good animal practice following the guidelines of the Institutional Animal Care and Use Committee of Utrecht Union. Swedish Research Council Grant 348-2005-5992: Conservation biology of Banteng and Bali cattle in Indonesia. Strengthening of research capabilities in Asia Link Project Reproductive biotechnology: modern technology to improve livestock production under traditional Asian conditions, European Union. SWeden Details and references for the DNA samples and methods used for the molecular analysis are provided in the Supporting Information file.
University. Blood and skin tissue samples from Bali cattle and banteng and blood samples from zebu breeds were collected on different locations (table 1). Blood and skin tissue samples from 8 bantengs were obtained from Ragunan Zoo, Jakarta. DNA was isolated by using standard SDS/proteinase K extraction [15] or the Qiagen blood and tissue extraction kit (Qiagen, Valencia, USA).

PCR-RLFP and sequencing

PCR-RLFP on a mitochondrial cytochrome \( b \) gene segment was carried out as described previously [16] with separate digestions by \( XbaI \) and \( TaqI \), indicating a zebu and banteng origin, respectively. In all cases, the separate digestions of mitochondrial DNA agreed and ruled out mistypings by genetic polymorphisms or failure of the digestions. PCR-RFLP of a Y-chromosomal \( SRY \) gene segment, in which a \( BfaI \) site indicates a banteng origin (table 2), was performed as described [16]. The absence of the \( BfaI \) site in undigested samples was confirmed by sequencing the same \( SRY \) fragment, which also indicated second banteng-specific mutation and differentiation between zebu and taurine origin [17]. For two samples a taurine origin was confirmed by sequencing intron 10 from the \( ZFY \) gene segment [17,18]. For these samples, an indel in the same intron [18] as well as genotyping by K-Bioscience (Hoddesdon, UK) of single-nucleotide polymorphisms (SNPs) in exon 11 of \( ZFY \) and in \( UTY \) [18,19] differentiated between the taurine Y1 and Y2 haplotypes. Table 2 summarizes the Y-chromosomal species variation and haplotype variation.

Microsatellite genotyping

Microsatellite analysis of the loci \( INRA63, INRA5, ETH225, ILSTS5, HEL1, INRA35, ETH152, ETH10, CSSM66, ETH3, BM2113, BM1824, HEL13, BM1918, ILSTS6 \) and \( CSRM60 \) was carried out using 10 ng of genomic DNA, 2 \( \mu M \) of M13 tailed forward primer, 10 \( \mu M \) of reverse primer, 10 \( \mu M \) of M13 oligonucleotide coupled to a fluorescent dye, \( Taq \) DNA polymerase and a standard PCR protocol. Fragments were separated on an ABI 3100 apparatus (Applied Biosystems, Foster City, CA). Allele size lengths have been standardized via comparison with a common reference sample. Data from Indian zebus are from ref. [20].

Data analysis

Checking of microsatellite data and calculation of expected heterozygosities was performed using the Excel-based microsatellite toolkit [http://animalgenomics.ucd.ie/sdepark/ms-toolkit/]. Nei standard genetic distances were calculated using the program Microsat [http://hpgl.stanford.edu/projects/microsat/]. NeighbourNet graphs were constructed by the program Splitstree.

| Table 1. Genetic constitution of Indonesian and Indian cattle breeds. |
|-----------------|-----------------|---------------------------------|---|---|---|---|
| Breed/population | sampling site   | samples                         | microsatellites |   |
|                  | country/isle    | location                        | males | females | genotypings | haplo | |                         |
| Ongole           | India           |                                | 32    | 64     | 0.64         | 06    | 10.8                  |
| Nellore          | Brasil          |                                | 27    | 53     | 0.63         | 06    | 10.6                  |
| Tharparkar       | India           |                                | 35    | 60     | 0.63         | 06    | 10.3                  |
| Red Sindi        | India           |                                | 35    | 52     | 0.63         | 06    | 10.6                  |
| Sahiwal          | India           |                                | 10    | 18     | 0.58         | 06    | 10.5                  |
| Desi             | India           |                                | 24    | 48     | 0.65         | 06    | 10.6                  |
| Hariana          | India           |                                | 10    | 18     | 0.68         | 06    | 10.6                  |
| Aceh             | North Sumatera  | Langsa                          | 8     | 16     | 0.68         | 06    | 10.8                  |
| Pesisir          | West Sumatera   | Painan                          | 17    | 34     | 0.65         | 06    | 15.7                  |
| Fillar Ongole    | South Sumatera  | Palembang                       | 5     | 10     | 0.78         | 07    | 16.3                  |
| Galekan          | East Java       | Trenggalek                      | 36    | 72     | 0.73         | 06    | 22.0                  |
| Madura           | Madura          | Bangkokal                       | 18    | 36     | 0.75         | 06    | 31.3                  |
| Bali cattle      | West Sumatera   | Sijunjung                       | 15    | 30     | 0.67         | 06    | 59.5                  |
| Bali cattle      | South Sumatera  | Palembang                       | 24    | 48     | 0.62         | 05    | 58.5                  |
| Bali cattle      | South–West Sumatera | Bengkulu         | 5     | 10     | 0.7          | 05    | 58.5                  |
| Bali cattle      | Sulawesi        | Kendari, Kanowe Selatan         | 9     | 18     | 0.64         | 05    | 56.5                  |
| Bali cattle      | Bali            | Denpasar, Tabanan               | 25    | 50     | 0.61         | 05    | 58.5                  |
| Banteng          | Java            | Ragunan zoo, Jakarta            | 6     | 12     | 0.37         | 03    | 39.3                  |

*Corresponding to the BfaI site in banteng.

Database entries and the numbering refer to sequences from taurine cattle.

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| Table 2. Y-chromosomal sequence variation diagnostic for indicine and Y1 and Y2 taurine haplotypes. |
|-----------------------------------------------|-------------|-----------------|---|
| gene | SRY | ZFY | UTY |
| Genbank entry | DQ336526 | DQ336536 | DQ336546 | AY936543 |
| Position | 2059 | 2100 | 2144 | 614 | 697–698 | 71 | 423 |
| Taurine Y1 | A | C | T | C deletion | G | G |
| Taurine Y2 | A | C | T | C | TG | T | T |
| Zebu | A | T | T | T | TG | T | T |
| Banteng | G | C | C | T | TG | T | T |

*Corresponding to the BfaI site in banteng.

Table entries and the numbering refer to sequences from taurine cattle.

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from the q value (Pritchard et al., 2000) after analysis with Indian zebus and Bali cattle as predefined clusters.

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(http://www.splitstree.org/, [21]). Model-based clustering was carried out using the program Structure (http://pritch.bsd.uchicago.edu/software.html , [22]), assuming admixture and correlated allele frequencies. Reproducible clustering was obtained after 30,000 burnin steps and 40,000 simulations. Clusters were either inferred or predefined as Indian zebu and Bali cattle, respectively. Results were displayed by the program Distruct (http://rosenberglab.bioinformatics.med.umich.edu/distruct.html, [23]).

Results

As indicated by specific PCR-RFLP assays and sequencing, the sampled Aceh and Pesisir zebras have zebu mitochondrial DNA, while maternal lineages from both species are represented in Filial Ongole cattle (figures 1 and 2). In two earlier studies, banteng mtDNA has been found in 20 out of 26 [9] or six out of seven [10] Filial Ongole animals. We found banteng mtDNA also in 56% and 94% of the East-Javanese Madura and Galekan zebu samples, respectively. However, the maternal origin of Bali cattle from five different locations on three isles is almost exclusively banteng with a zebu origin found for only 1 out of 125 sampled animals. This is in contrast to the mixed maternal origin of Bali cattle from Malaysia [13], but agrees with the results obtained for a feral population of Bali cattle [24].

Interestingly, Y-chromosomal typing as a probe of the paternal lineage does not completely parallel the mtDNA results (figure 1 and 2). All zebu bulls carry exclusively zebu Y-chromosomes. Only female Filial Ongole animals were sampled, but in another study [10] seven bulls from this breed were found to carry zebu Y-chromosomes. Zebu Y-chromosomes were also found in the East-Javanese Galekan and Madura breeds. However, for two Madura bulls the sequence of the ZFY and SRY gene segments (table 2) indicate a taurine origin, possibly resulting of experimental crosses with Danish Red and French Limousin bulls [8]. These two European breeds carry different Y-chromosomal haplotypes (Y1 and Y2, respectively, [19]). Different diagnostic SNPs (table 2) revealed that both Madura bulls with taurine Y-chromosomes carried the Y2 haplotype, compatible with a Limousin origin. The parental origin of Madura cattle may also depend on the sampling site, because we previously found banteng Y-chromosomes in two Madura bulls from a breeding station in Malang on Java [14]. With one exception in South Sumatera, all Bali cattle in our study descend from banteng bulls.

For estimation of the autosomal species composition, we genotyped 16 microsatellite markers from the panel of 30 microsatellites recommended by the FAO for diversity studies (http://lprdad.fao.org/cgi-bin/getblob.cgi?sid = -1,50006220) and compared the data with genotypes for the same markers from seven Indian zebu populations [20]. As indicated by the expected heterozygosity, genetic diversity of Indonesian cattle compares to that of Indian (table 1). In Bali cattle observed heterozygosity is clearly lower than the expected values, presumably because of inbreeding within local populations. However, clearly higher heterozygosity values were observed for the Indonesian Ongole, Madura and Galekan, while the lowest value found for eight wild bantengs probably indicates inbreeding in a zoo population.

Allele distributions (not shown) of Indonesian and Indian breeds also matched well. However, for several markers additional alleles also present in Bali cattle were observed. Quantitative species components were estimated by two different methods (see [25]). First, Nei genetic distances were visualized in a Neighbor Network (figure 3), Indonesian Aceh, Pesisir and Filial Ongole are close to the Indian zebu breeds, but are intermediate between Indian zebu and Bali cattle. Interestingly, Madura and Galekan cattle, several of which carry banteng mitochondria, are more distant from the Indian zebu and closer to Bali cattle. The different Bali cattle populations appear to be identical. Adding genetic distances to the captive banteng population links this population to Bali cattle with a distance that probably corresponds to their inbreeding (not shown).

Second, unsupervised model-based clustering, i.e., without prior information on ancestral clusters, [22] identified a zebu and a Bali cattle cluster (figure 2). Assuming that Indian zebu and Bali cattle are the ancestral populations we then carried out supervised clustering in order to estimate the levels of introgression via the individual membership coefficients (figure 2, table 1). For Aceh, Pesisir and Filial Ongole banteng introgression is in the range of 11–16%, but this is clearly higher for Galekan (22%) and Madura cattle (31%).

Discussion

Domestication of banteng probably took place around 3500 years BC [6,8]. Bali cattle is currently the main representative of the domestic banteng, and it is kept on several Indonesian Isles.
cattle population in Pandaan on East Java is also supposed to originate from banteng (T. Susilawati, Malang, pers. comm.). There is no reliable dating of the entry of the more common cattle species, which in Indonesia was very likely predominantly of zebu origin ([26,27]). Singalese immigrants may 1500 years ago have brought Indian cattle ([8]). Statues of a humped bull on Javanese Hindu temples evidence the presence of zebras in the 10th century. Photographs of original Javanese cattle from the beginning of the 20th century ([28,29]) also show humped cattle and resembled Madura cattle. However, starting by the end of the 19th century, imported Ongole zebras were more and more used for breeding on Java and other Indonesian isles, but not on the isles of Madura and Bali [6,8,28].

The predominance of zebu mitochondria in the Indonesian zebu breeds shows that not only zebu bulls but also zebu cows were imported. This is in contrast to the zebu populations in Africa and America, which emerged by systematic crossing of imported zebu bulls with taurine cattle [4,5]. Banteng mitochondrial DNA in Indonesian Ongole populations as well as the autosomal microsatellite analysis indicate crossbreeding of zebu and local cattle with a banteng maternal origin.

The Eastern Java Galekan cattle are supposed to descend from original Java cattle (T. Susilawati, personal communication). DNA analysis indicates that it descends from banteng cows and zebu bulls. Madura has long tradition of well managed cattle husbandry [28,30,31]. The Madura breed predates the import of Ongole bulls and Madura bulls were used for crossing on East Java before the import of Ongole bulls [28]. DNA analysis of Madura cattle shows a combination of a zebu paternal lineage with a mixed zebu-banteng maternal origin. Experiments of upgrading the local cattle with Danish Red and Limousin taurine bulls, which have similar coat colors [8] were not pursued, but apparently left taurine Y-chromosomes in the Madura population. Although indiscriminate cross-breeding with exotic breeds is a major threat to the conservation of genetic resources, it is also evident that introgression of foreign material at such a low level in this case did not affect the identity of the breed.

Our data further indicate that Bali cattle on different locations in Indonesia has been kept separate from zebu, this in contrast to mixed zebu-banteng Bali cattle populations from Malaysia [13].

Evidently, the history and breeding of Indonesian cattle has resulted in a unique genetic resource that combines the general tolerance of zebu to tropical and dry climates with the adaptation of domestic banteng to Indonesian conditions and husbandry. Information about the history and species composition as reported here appears most essential for strategic choices regarding breed management and conservation. Furthermore, the adaptation of Indonesian cattle to different modes of management under tropical conditions may very well be exploited outside Indonesia, especially if the high-temperature zones expand because of current global climate trends [32].

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Author Contributions
Conceived and designed the experiments: KM GA HRM BP RWP BC JAL. Performed the experiments: KM MO HTAVT SM BHV. Analyzed the data: KM MO HTAVT SM BHV GA JAL. Contributed reagents/materials/analysis tools: KM HRM BC JAL. Wrote the paper: KM GA HRM BC JAL.

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