Reproduction and genetic diversity of the swamp buffalo

Yindee Marnoch

The water buffalo is an integral component of traditional Asian agriculture through contributions to milk, meat and draft power [39] and thus has a pivotal role in agriculture in several Asian countries [44]. As indicated by their species name, water buffaloes frequently wallow in water to cope with the hot climate. They are kept mostly on small farms as they can utilize relatively poor digestible feeds and provide milk, meat, hide and draft power. Indeed, in India, one of the world's top producers of milk, more than half of the milk is produced on small farms by buffaloes and not by dairy cattle. Such farms constitute a significant part of agriculture and 60-80% of the Asian population is affiliated with farming. As the human population increases the buffalo holds great promise and potential for meeting the required milk and meat production [40].

The Asian buffalo includes three species, the Indonesian Anoa (*Bubalus depressicornis*), the Philippine Tamarau (*Bubalus mindorensis*), and the water buffalo (*Bubalus bubalis*), which is derived from the wild buffalo (*Bubalus arnee*). The water buffalo includes two subspecies: the river type (*Bubalus bubalis bubalis*; 2n=50) and the swamp type (*Bubalus bubalis carabensis*; 2n=48), which were domesticated approximately 5000 years ago and are interfertile [70]. The swamp buffalo in China and Southeast Asia is mainly used as draught animal, while more to the west the river buffalo (South- and Southwest Asia, Egypt, Balkan, Italy, South America, Caribbean) is a dairy animal.

Figure 1. (A) Swamp buffaloes have swept back horns and are native from Southeast Asia to China. (B) River buffaloes generally have curved horns and are native from India to Middle East Asia.

Although the total world buffalo population has increased from 120 to 160 million in the last two decades, the number of swamp buffaloes has declined. This is due to mechanization of agriculture, increased meat demands of the human population, increased urbanization and poor reproductive performance [63]. Indeed the lack of knowledge of swamp buffalo reproduction is a major concern. The major problems in buffalo reproduction are an inherent late maturity, a lack of obvious estrus behavior, a prolonged inter-calving interval and a distinct seasonal breeding pattern. Modern reproductive technology, such as artificial insemination (AI), in vitro fertilization (IVF) and embryo transfer (ET), which are routinely applied in the dairy cattle industry, have to be improved for and adapted to the buffalo [72]. Assisted reproductive techniques (ART) are essential for a wider use of superior sires selected for specific traits as milk production, meat quality and longevity to increase efficiency of reproduction and production.

Another avenue of improvement will be a more systematic and planned genetic management. So far selection of buffalo sires was not focused on productivity as many superior bulls with aggressive behavior were culled and subdominant ones used for breeding. A more effective genetic management should not only consider productivity, but also guard against inbreeding in closed populations and an overall loss of the genetic diversity. This is essential in order to meet future production needs in various environments, to allow sustained genetic improvement and to facilitate adaptation to changing breeding objectives. In addition, molecular studies of livestock genetic diversity allow a reconstruction of the history of the species, including the domestication process, the divergence of river and swamp buffalo and later demographic events.

The swamp buffalo in Thailand

The swamp buffalo is the indigenous buffalo in Thailand, called “Ai-Tui” for “honest royal worker” and is, together with the elephant, a national symbol. The Thai buffalo population has declined from about 5.5 million in the seventies to 1.8 million in 1999 (Figure 1), currently is 1.3 million and the buffalo may become extinct in Thailand if this trend will continue. This reduction is caused by (1) the decrease in rice-producing areas, (2) urbanization and industrialization of Thai rural areas, (3) the replacement of the buffalo by tractors, (4) the castration of the strongest bulls [43] and (5) inefficient reproduction as illustrated by the long days open: the long period it takes for the animal to be mated again and get pregnant after parturition. Most Thai buffaloes belong to small-scale farmers who raise the animals in their backyard. The average herd size is five to ten head of buffalo per family and profits are only marginal.

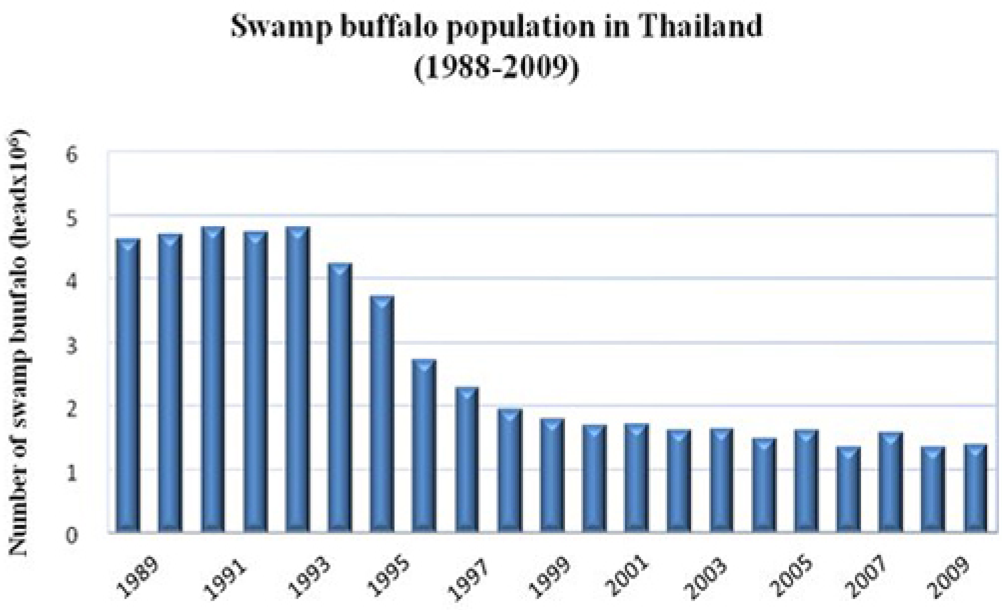

Figure 2. Number of swamp buffalo in Thailand

Female reproduction

Buffalo breeding under village conditions is poorly managed in Thailand. During the traditional plantation season (rainy season; July-October) buffaloes are tied up and fed with rice straw for a period of approximately four months, thus losing the opportunity to breed. After the harvesting period (winter season; November-January), farmers allow the buffalo to graze, which leads to spontaneous unregistered breeding [62]. Calving may occur throughout the year, but happens mostly during the end of the rainy season (July to September) and the winter season (October to January). Since the gestation period is 9 months, the most optimal mating period starts in October but the dry or summer season (February to June) is less suitable for mating [79]. Seasonal stress with suboptimal nutrition appears to be responsible for a long anestrous period in the buffalo [5] [59] [74]. However, a distinct seasonal reproduction pattern is also observed in Italian buffaloes on a well balanced, constant diet [87]. Environmental conditions, both tropical and subtropical, may be of significance for the inter-calving period, since calving during the unfavorable season might result in ovarian inactivity until the breeding season and be a major cause of buffalo reproductive inefficiency. The long postpartum period (days open) in Thai swamp buffaloes averages 18.3 month [38]. It remains to be determined whether ovarian inactivity in the postpartum period is indeed the main cause or, as in cattle, lack of subsequent normal follicular development and atresia of the dominant follicle are the primary factors that affect reestablishment of ovarian cyclicity [17]. In suckled buffaloes, a relatively slow growth rate of the ovulating dominant follicle has been reported [33]. Postpartum anestrus is a common phenomenon in high yielding dairy cattle at a negative energy balance. Suboptimal (detection of) estrus behavior can however also occur in animals with active ovaries containing developing follicles that produce steroids. Indeed, under tropical conditions and heat stress, the efficiency of estrus detection can be extremely poor, resulting in unobserved estrus. It is important to distinguish between suboptimal ovarian activity, silent ovulation (lack of overt signs of estrus) and unobserved estrus (poor estrus detection efficiency), which can greatly increase the incidence of a registered anestrus. Follicular dynamics or lack thereof could be evaluated by ultrasound monitoring of the ovary combined with measurements of progesterone. This would be a valuable tool for adequate management of postpartum anestrus through e.g. nutritional management or hormonal treatment. Postpartum anestrus is indeed a major cause of poor reproductive performance in buffaloes [86], and reproductive efficiency can be improved by pharmacologically managing uterine involution [68] or stimulation of the pituitary-gonadal axis by e.g. GnRH or PGF2α [17].

The length of the estrus cycle (19.9 ± 4.4 h; [49]) is in the swamp buffalo slightly longer than in the river buffalo (23.8 ± 6.2 h; [41]). The signs of estrus described as bellowing, vulvar swelling, mucous discharge, decreased milk yield and standing heat [11] [41] [61] [69] are relatively difficult to detect compared to detection in cattle as these signs are weakly expressed. The estimated time interval between the end of estrus and ovulation is reported to be approximately 13.9 h in the swamp buffalo [49] and 11 h in the river buffalo [53]. However, timing of (artificial) insemination is difficult because silent heat does often occur, indeed, one of major problems for buffalo reproductive efficiency.

Timing of insemination is dependent on the time of ovulation. The physiological control of recruitment, selection, growth, dominance, ovulation and atresia of ovarian follicles in the postpartum swamp buffalo is not well understood and such knowledge is essential [1] for amelioration of reproductive efficiency. Insight in these phenomena may help to optimize reproduction by e.g. adaptation of management regarding signaling of estrus behavior in case of ovarian activity or hormonal treatment to induce follicular development when ovaries are inactive, although this treatment needs to be optimized [21] [56] [67] [76].

Figure 3. (A) Bull swamp buffalo (left) is smelling cow (right) (B) Bull wrinkling his nose and curling his lip, signs of the cow (right) being in estrus.

The use of real time ultrasound imaging has considerably increased the knowledge of follicular wave development in cattle, which is a key factor in the application of assisted reproduction [28]. Although much knowledge of follicular wave dynamics in cattle is available, little is known about follicular dynamics in the swamp buffalo [80].

Figure 4. (A) Use of ultrasound imaging to monitor folliculogenesis without affecting reproductive performance (B) Mucus discharge after rectal palpation was observed in most of the female swamp buffaloes in estrus.

Assisted reproduction

Assisted reproductive technologies have become essential livestock breeding tools. Various techniques have been developed to increase the number of offspring from superior animals including AI, sperm sexing, multiple ovulation and embryo transfer (MOET), embryo cryopreservation, ovum pick-up (OPU), in vitro embryo production (IVP), and gamete and embryo banking. These techniques are routinely applied in cattle, especially for bull selection but, apart from AI, are rarely used in buffalo breeding [6]. Knowledge of follicular development is important for ART but is lacking for the swamp buffalo. In particular, considerable differences in reproductive traits among different breeds of buffaloes exist [24] and data from river buffaloes are not directly applicable to swamp buffaloes. Indeed, swamp buffaloes are less productive [63] and also inefficient breeders compared to river buffaloes due to the inherent susceptibility to environmental stress, which causes anestrus and sub-estrus [5]. As mentioned above, bulls can have a great impact on genetic improvement through artificial insemination. Superior females can have their impact as bull mothers, especially through an embryo transfer program using various semen donors.

Superovulation and ET have firstly been applied in the buffalo approximately three decades ago. However, compared to results in cattle, superovulation and embryo recovery are suboptimal in the buffalo because of a poor superovulation response [56] or of an insufficient availability of follicles combined with a high rate of atresia among the growing follicles [15].

Ultrasound-guided transvaginal follicle aspiration (ovum pick-up; OPU) can be used for the efficient recovery of cumulus-oocyte complexes (COCs) for embryo production in the cattle (*Bos taurus*) [18] [19] [35]. This technique has been applied in many species including goat [45], zebu, river buffalo [7] and swamp buffalo [26].

The yield of OPU depends on the pool of antral follicles available for aspiration. Ovarian follicles develop in a wave-like pattern; hence the numbers of antral follicles vary during the cycle. Most studies concern cattle, but because of follicular dynamics differ between species the data from these studies cannot be extrapolated to the buffalo [83]. Moreover, buffalo ovaries are on average small in size [51] and as a consequence OPU in swamp buffaloes is a relative difficult procedure compared to OPU in cattle. For an efficient OPU-IVF program the quantity and quality of oocytes collected is a key factor. The number and developmental status of ovarian antral follicles, and thus the potential number of oocytes to be harvested is affected by the length of the interval between aspirations. OPU in cows at short intervals usually results in recovery of numerous, good-quality COCs. Data on OPU in the swamp buffalo refer to e.g. bi-weekly collections. Thus follicular development and oocyte quality when OPU is performed at shorter intervals need to determine in swamp buffaloes as well. In addition, performance of buffaloes under tropical conditions needs to be characterized as long-term exposure of donors to heat stress can have a delayed deleterious effect on ovarian follicular dynamics and oocyte competence [42]. So far, most studies have been performed on river buffaloes under less stressful climate conditions.

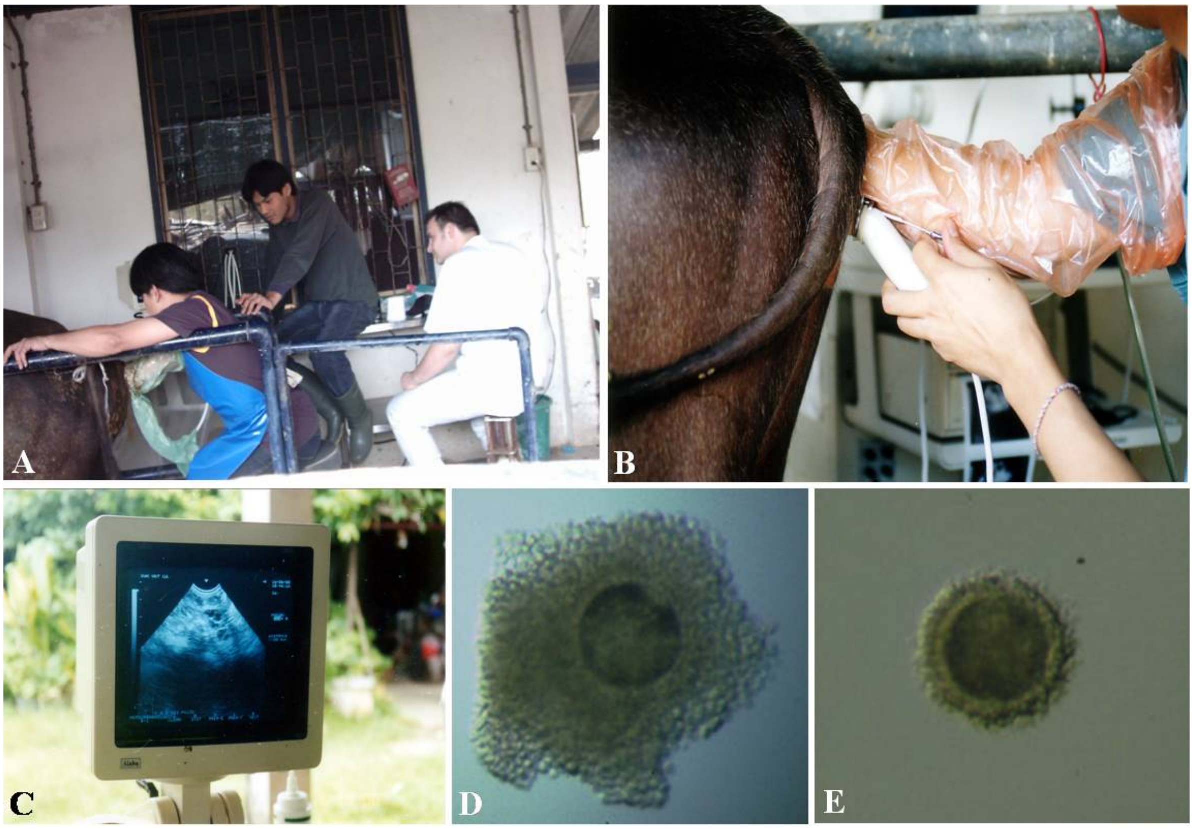

Figure 5. Ovum pick-up technique for oocyte collection from live female swamp buffalo. (A) The cow is held in the enforcement case that facilitates rectal palpation. (B) An ultrasound probe with a long needle connected to a vacuum pump is passed through the vagina while the other hand holds the ovary by rectal palpation. (C) Real time ultrasound image. (D) Aspirated cumulus oocyte complex (E) Oocyte with incomplete cumulus cell layers.

Oocyte quality can be assessed by morphological evaluation and by the developmental competence of collected cumulus-oocyte complexes (COCs) following maturation and fertilization in vitro. Adequate presence of cumulus cells is necessary for cytoplasmic and nuclear maturation in cattle [85] and disruption of cumulus cell layers or partly denudation will reduce developmental competence [60]. An integral aspect of in vitro embryo evaluation is a successful maturation of the oocyte. Complete nuclear maturation encompasses the progression of the oocyte from the meiosis I to the meiosis II stage and extrusion of the first polar body, requiring proper meiotic spindle formation. Adequate cytoplasmic maturation is also important, for example redistribution of the cortical granula, is also essential for a timely cortical reaction subsequent to fertilization and prevention of polyspermia.

Genetic management

General considerations

Analysis of the pattern of genetic variation is not only essential for genetic management, as in other livestock species, but may also reveal the genetic history of the species. At the molecular level, genetic variation can be evaluated by three complementary approaches [10]:

(1) Analysis of the diversity of maternal lineages by comparison of mitochondrial DNA (mtDNA) sequences. In livestock species mtDNA is particularly informative about relationships with wild ancestor species, time and place of domestication and the relation between subspecies, as the river and swamp buffalo (see below), and between buffalo populations of different subcontinents [10].

(2) Analysis of paternal lineages by investigating the Y-chromosomal variation, which is particularly informative about gene flow between populations by male introgression.

(3) Autosomal variation by genotyping genetic markers on the autosomes. So far microsatellites are the most popular markers for livestock, but these are being superseded by SNPs. Autosomal variation can be correlated with traits in linkage analysis and association studies, applied in routine tests of individual identification or paternity or used for studying patterns of diversity. It reveals relation between breeds and is most informative about recent genetic events, as expansions, migrations, population bottlenecks, introgression and genetic isolation. This also provides tools for genetic management. For instance, an excess of homozygosity is diagnostic of inbreeding, which increases

disease susceptibility and decreases reproductive fitness [36] [66] [81].

The wild water buffalo

The wild water buffalo is a highly endangered species. Although the population of these animals is estimated to be 4000 by the FAO, possible less than 200 or even no purebred wild animals exist [72]. Small isolated populations may remain in the Kosi Tappu Wildlife Reserve in Nepal, Bastar and Raipur Districts of Madhya Pradesh in India, Royal Manas National park (Bhutan), and Huai-Kha-Khaeng Wildlife sanctuary in Thailand [72]. Conservation management plans need to be developed for purebred wild populations while a low level of domestic genetic introgression is probably unavoidable.

Figure 6. Photograph of a wild buffalo using camera trapping in the Huai-Kha-Kheang wildlife sanctuary. The population of wild buffaloes in Thailand is estimated to consist of only 40-50 animals.

Evolution and biological diversity of the buffalo

Molecular studies so far have focused on mtDNA and microsatellites. Analysis of maternal lineage via mtDNA resulted in two alternative conclusions on the origin of river and swamp types and their relations with the wild buffalo. (1) According to some authors [50], the river type was domesticated in India, after which the swamp type emerged by introgression of wild water buffalos in China and Southeast Asia [50]. (2) According the most commonly held view, there were separate domestications in China and India of swamp and river types, respectively [12] [13] [14].

The estimated of the time of divergence of river and swamp type varies widely between studies: from 1.7 million years ago (mtDNA cytochrome b, [25], 1 million years ago (mtDNA, [31], 28,000-87,000 years ago (mtDNA D-loop, [13]) and 10,000-15,000 years ago using biochemical markers [2]. Interestingly, the native buffalo of Sri Lanka, has morphological characteristics of the swamp type, but the chromosome number and microsatellite loci of the river type [2] [13].

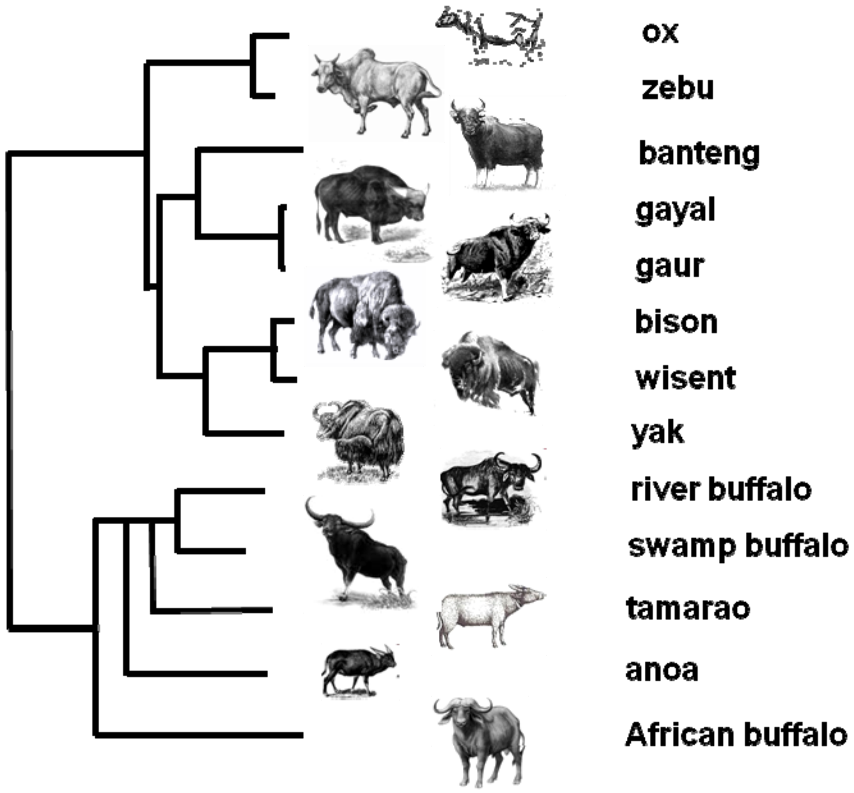

Figure 7. Evolutionary tree of the bovidae subfamily including the swamp buffalo.

In the swamp type, two separate maternal lineages A and B have been identified with an estimated divergence time of 18.000 years. Lineage A is the most common, but geographic distribution patterns have only been studied in China.

Genetic differentiation of swamp buffalo populations by breed formation is lower than for river buffalo and other livestock. Molecular studies revealed that only 2.8% [30] of the genetic variance of Chinese water buffaloes is accounted for by differences between populations. Higher values have been reported for river buffalo (3.4%, [12]; 9.7%, [84]), cattle (10-11%, [54]), horses (8%, [37]) and pigs (13%, [58]).

The Thai swamp buffalo

Although Thailand used to have the second largest number of swamp buffaloes in the world after China, the swamp buffalo population drastically declined from 4.7 million in 1990 to 1.4 million in 2002. A selective breeding program (1983-1993), which aimed at lowering the age at first calving, reducing the calving interval and increasing growth rate [77] did not halt this decline and production and reproduction is still inefficient. Characterization of genetic diversity may support a systematic genetic improvement and also help to prevent inbreeding. Inbreeding may especially occur in the small wild populations of buffaloes, which are confined to a fragmented habitat area. An effective management program of farm animal genetic resources requires characterization of the various populations, of their geographic distribution, of the production environment, and of genetic relationships of the different populations.

So far, hardly any studies have been done on the genetic characterization of the swamp buffalo in Thailand. Y-chromosome variation is a most powerful marker for phylogeography and speciation in within a species [65], but when we started our studies no Y-chromosomal genetic markers were available for the water buffalo. MtDNA as marker of the maternal lineage heritage has been studied mainly outside Thailand. So it is not at all clear how diverse the Thai swamp buffalo mtDNA is in comparison with e.g. the Chinese population [14]. Based on these outcomes, a breeding program for preservation of the biodiversity of the Thai swamp buffalo should be developed.

The objectives of our studies

To determine female reproductive physiology and behavior in domestic swamp buffaloes in Thailand, where the climate, ecology and nutritional are close to that of the wild population. For efficient breeding, adequate timing of copulation and insemination is essential. Signs of estrus behavior will be evaluated in the swamp buffalo for optimizing estrus detection methods that can easily be executed by small farmers. This is combined with a follicular wave study using ultrasonography in the postpartum period with the ultimate aim to decrease the long period of days open and thus improve reproductive efficiency. The ART method OPU was chosen for further study as a way to improve the swamp buffalo genetics via the maternal line. Estrus behavior is studied by three methods (mucus appearance, personal and closed circuit camera observation) to evaluate timing of estrus. This study is combined with ultrasonographic observations of the postpartum ovary to evaluate follicular development and selection and thus define the breeding time.

In vivo and in vitro swamp buffalo oocytes are assessed for quality as well as quantity. Oocytes were collected from live animals by OPU, comparing once and twice-weekly oocyte collection sessions. In addition ovaries were collected after slaughter from pregnant and non-pregnant donors to evaluate the consequence of origin on in vitro oocyte quality. Analysis of genetic relationship between different swamp buffalo populations in Thailand and elsewhere and, in a wider context, between the river, swamp and wild buffalo, in order to enable maintenance of genetic diversity of buffalo populations. We focus on the Y-chromosome and mitochondrial DNA as markers of the paternal and maternal lineages, respectivelyY-chromosomal variation within Thailand is evaluated. This also allows a comparison with the river type Y-chromosome. The mitochondrial haplotype diversity is analyzed and compared with Chinese swamp buffalo.

Summarizing discussion

The water buffalo is one of the most important domestic animals in Southeast Asia. In Thailand it is, together with the elephant, a national symbol. Until 2 decades ago, Thailand harbored the largest buffalo population after China. Its draft power has been of high significance for agriculture and Thai farmers used bulls to prepare the land to plant rice. The buffalo has also been used as transportation animal (horses are rarely used in Thailand for this purpose). Since the introduction of the tractor the Thai buffalo population has rapidly declined because most Thai farmers prefer to use machines. Furthermore, since the most sturdy animals also tend to be relatively aggressive and difficult to restrain, many farmers had such animals castrated to optimize animal handling and for the farmer's safety. Consequently, relatively small bulls were used for breeding and the Thai buffalo gradually became small in body size. As in the last two decades the Thai population increased from 30 to 60 million persons, the demand for buffalo meat did rise and more buffaloes were slaughtered while breeding was relatively unsuccessful. It is now the policy of the Thai government to increase buffalo production, to improve the reproduction and to conserve the diversity of available genes. However, this is hampered by a lack of knowledge of various (sub) species specific aspects of swamp buffalo reproduction. Moreover, there is only limited insight in the level and pattern of genetic diversity, while molecular tools for genetic management are not available for this species.

Female reproduction: a general perspective

The buffalo can be used for draft power and transport, and is particularly known for its capacity to thrive on roughage that would be considered substandard or even not suitable for cattle. Buffaloes are well adapted to poor pasture quality and swamp areas where cattle cannot be kept. Moreover buffaloes have better resistance to tropical climate conditions and are more resistant to tropical diseases. Buffalo breeding is poorly managed in Thailand. After 300-340 days of pregnancy, most swamp buffaloes give birth at the end of the rainy season or in the winter season which also is the harvesting period in Thailand. During the harvest - a period of approximately four months - buffaloes are tied up if they are not at work thus losing the opportunity to breed. In the following period - the dry or summer season - temperatures are relatively high and nutrition is suboptimal, which hampers efficient reproduction.

Assistance Reproductive Technology (ART) may help to improve the buffalo reproductive performance e.g. through artificial insemination (AI), a tool that also facilitates the optimal use of genetically superior sires. AI requires an adequate timing of insemination and the factors involved in this process such as heat detection and follicular development have been investigated [29]. Follicular wave development was studied in the postpartum buffalo using ultrasonography with the ultimate aim to reduce the number of days open. The contribution of genetically superior females in breeding programs can be enhanced through embryo transfer. Multiple ovulation and embryo transfer (MOET) has been applied in the buffalo, but typical results were poor when compared to those in cattle, due to a low response of the ovary to drugs that would stimulate multiple ovulation. Hence, ovum pick up (OPU) offers a possibility to recover higher numbers of oocytes from genetically superior donors for subsequent in vitro maturation and fertilization followed by embryo transfer. Transvaginal ultrasound-guided follicle ablation was carried out as a method of synchronizing follicular wave emergence starting at a random stage of the estrous cycle without the need of hormonal pretreatment, followed by ovum pick up at appropriate intervals. We investigated the oocyte quality, a key factor in in vitro fertilization (IVF) procedures, following in vitro maturation of oocytes obtained in vivo or in vitro [29].

Estrus behavior and timing of mating

Failure to detect estrous is a major problem that hampers breeding efficiency in buffaloes [47]. Various management factors contribute to suboptimal estrus detection, and the inability to recognize the signals characteristic for estrus is a common causal factor. Detection of estrus is essential for timing AI and thus crucial for efficiency of buffalo breeding programs by allowing a wider use of genetically superior bulls. We investigated estrus behavior in the postpartum female buffalo by evaluating her interaction with herd mates and with a bull [29]. An estrous cow will show standing heat, raise interest from the male and induce his sex specific behavior such as flehmen [29]. For optimal registration, closed circuit camera recording was used for 21 hours a day in addition to twice daily personal observation. Morphological changes of the female genital apparatus were monitored daily and combined with ultrasonography to evaluate ovarian activity.

Our study demonstrated that the closed circuit camera observation is an excellent and efficient tool for estrus detection due to its high sensitivity and efficiency compared to other methods [29]. This is supported by results from a previous study in cattle [4]. However, most local farmers cannot afford such expensive equipment, leaving personal observation as second best. This can optimally be performed early in the morning, between 6 and 8 AM, when the temperature is relatively low and weather conditions less stressful than later during the day. This is in agreement with conclusions from an earlier study [78] which reported that buffaloes showed more estrous activity in the morning (6.00-7.30 am) than in the afternoon (2.00-3.30 pm) or during the night (11.00-11.30 pm). We demonstrated that the presence of a buffalo teaser bull is a very helpful tool to diagnose the heat of the female swamp buffalo. However, small buffalo farms do not have a teaser bull available. Other signs of estrus of the female buffalo are sniffing of the vulva of other females, wrinkling her nose while curling her lip, similar to the flehmen behavior of the bull, and mounting other females. Estrus behavior can easily go undetected; hence at least one person on a farm should be skilled in estrus detection methods.

Efficiency of reproduction in the postpartum period

Prolonged postpartum anestrus is one of the major causes of poor reproductive performance in buffalo, leading to the long period of days open and thus a long calving interval [86]. Pharmacological treatment may reduce the time required for uterine involution and thus immediately improve postpartum buffalo reproductive efficiency [68]. Nevertheless, insight in ovarian activity during the postpartum period is required to improve reproductive efficiency, especially when refraining from medical intervention. Ovarian function in primiparous and pluriparous postpartum river buffaloes has been studied in a sub-tropical environment [20]. No significant differences between primiparous and pluriparous postpartum animals were observed, but buffaloes were prone to a seasonal anoestrus. Most studies have been performed on river buffaloes, in various reproductive conditions such as limited versus unlimited suckling [27] [46] [64] [75] [82]. Considerable differences in reproductive traits between swamp and river buffaloes exist [24], while productive traits that affect reproductive efficiency (e.g., milk production) also significantly differ between river and swamp buffaloes. As conclusions from studies on river buffaloes cannot be directly applied on swamp buffaloes and climate conditions also markedly influence reproduction, investigations on reproductive efficiency in the swamp buffalo under tropical conditions are required.

Multiparous postpartum swamp buffaloes were evaluated between 5-9 d to 94 d postpartum using ultrasonography [29]. After parturition, follicular growth in swamp buffalo showed a wave-like pattern with 1-2 waves per estrous cycle. The growth rate and size of the first postpartum ovulating follicle were similar to those in the Italian river buffalo [20]. However, the mean interval from parturition to the first postpartum ovulation [29] was longer than in the Italian buffaloes [20] but remarkably shorter than in limited suckled Nili-Ravi buffaloes [82] and suckled buffaloes [13] [14] [48]. Silent heat and short estrus cycles [29] between the 1st and 2nd ovulation were common in our study and also reported for milked buffalo [27]. Follicular dynamics in most sucking swamp buffaloes become standardized after the second ovulation (13/16, 81.3%, [29]).

To improve postpartum buffalo reproduction efficiency, proper husbandry management such as quality of feed, optimal estrus detection and a fertile bull are crucial. In our study, the postpartum buffalo could be successfully mated without hormonal treatment within 2 months after parturition at the second spontaneous ovulation. Adequate estrus detection methods are essential for evaluation of cyclicity. Our study was performed during the winter period. Although the temperature in Thailand does not differ much between seasons, a possible annual pattern of ovarian activity in the swamp buffalo and thus of the estrus activity of Thai swamp buffalo should also be investigated.

Efficiency of oocyte collection by ovum pickup

Another way to improve the reproductive efficiency and optimally exploit the genotypes of superior cows is by embryo transfer. Multiple ovulation and embryo transfer (MOET) have been attempted in swamp buffaloes but this was less successful than in cattle [55]. However, IVP of transferable embryos via ultrasound-guided transvaginal follicular puncture OPU and subsequent IVF have been reported to be more efficient in this species than the procedures for in vivo production of embryos [32] [71]. Pre-puncture hormone treatments have been applied in cattle to stimulate follicular growth and thus increase oocyte yield during the puncture session [3] [8] [9] [34] [73]. Hormone stimulation followed by OPU has also been applied in swamp buffaloes [26], but the response decreases significantly if hormonal stimulation is prolonged [56]. An alternative method through repeated follicle puncture during several weeks appeared to be an effective treatment that avoids expensive FSH-treatments [23]. We investigated the applicability and efficiency of this method [29].

Non-lactating multiparous Thai swamp buffaloes were tested as oocyte donors using transvaginal ultrasound-guided follicle ovum pick up as collection method while refraining from hormonal stimulation. Follicles were punctured twice a week for 10 consecutive sessions immediately followed by follicular puncture once a week for 10 consecutive sessions. Due to the small size of buffalo ovaries [51], OPU in swamp buffaloes is a relatively difficult procedure compared to OPU in cattle. The majority of follicles were of small size (<4 mm), independent of the frequency of OPU, similar to what has been described for bovine [9]. When OPU was performed once weekly, follicle size had significantly shifted from medium sized (5-8 mm) to large (>9 mm). The oocyte recovery (0.8/session) was somewhat lower than that described by [57] in river buffaloes (1.2/session) in twice weekly sessions. However, the weekly harvest of oocyte in twice weekly session was higher than in once weekly sessions [29]. Seasonal differences (mainly winter season versus mainly rainy season) might underlie this variation as season has been described to influence oocyte recovery rate, being highest in the breeding season [57]. The percentage of oocytes from the once weekly OPU that reached metaphase-II was 51.4% [29]. In contrast, the percentage of oocytes that reached the metaphase II stage was significantly higher when oocytes were collected twice weekly (65.7%) indicating improved quality of oocytes in this group as also reported in cattle [52]. This finding is in agreement with the hypothesis that a dominant follicle, emerging approximately 3 days after OPU will exert a negative effect on developmental competence of oocytes of subordinate follicles and thus on the oocytes collected during weekly sampling [60]. There was no difference regarding the side of ovary punctured either twice or once weekly and right or left side of ovaries, similar to results obtained by [26] who investigated prepubertal swamp buffalo calves after FSH superstimulation. Our study indicates however that oocytes for IVM can successfully be collected by ovum pick up (OPU) in the swamp buffalo, without hormonal pretreatment, and two collections per week result in better quality oocytes than once per week.

When oocytes were collected via follicle puncture of ovaries following slaughter, most follicles were small (<4 mm) [29]. Morphological characteristics of oocytes did not differ significantly between follicle size classes as was found in river buffalo [22]. Developmental competence of oocytes from medium sized follicles, expressed a percentage reaching the MII stage, and was higher than the competence of oocytes from small size follicles. Reproductive state (pregnant versus non-pregnant) had no major influence on oocyte quality. Morphological abnormalities are more frequently encountered in oocytes obtained from in vitro than in those collected in vivo. This is not surprising as animals to be slaughtered encompass a higher percentage of sub-fertile and aged individuals, characteristic for a bias to reduced oocyte quality. Moreover, post mortem deterioration could affect oocyte quality when adequate preservation conditions cannot be met under the tropical conditions.

Genetic diversity in the swamp buffalo

In the last two decades the water buffalo population in Thailand has declined. Demographic data are essential to assess the risk status of the buffalo population. There are three major risk factors:

(1) A small effective population size, which depends on the number of males and females indeed used for breeding, may lead to inbreeding.

(2) The current and predicted future downward population trend with a concomitant loss of genetic

diversity.

(3) The geographic distribution of the buffalo with in concentrated populations a higher vulnerability to disasters such as plague.

In order to support a more effective management of the buffalo genetic resources, we studied the genetic diversity of both the paternal and maternal lineages. This is also relevant for a reconstruction of the genetic history of the water buffalo. As marker of the paternal lineage, Y-chromosome variation is informative on introgression, phylogeography [16] and speciation [65]. However, there were so far no data available on Y-chromosomal haplotypes for the water buffalo. In our study, we collected 21 native domestic Thai swamp buffalo bulls [29], two offspring of a Thai wild bull and a domestic cow and 7 river buffalo samples from Italy, India and Sri Lanka. Sequencing of 4663 bp from the water buffalo Y-chromosome in this study confirmed that the paternal lineages of the swamp and river buffalo originate from separate wild parent populations. This, in line with the mtDNA variation as marker of the maternal lineage studied previously [12] [13] [14]. The separate domestications of two subspecies are not without precedent: taurine and domestic cattle were domesticated in Southwest Asia and the Indus valley, respectively, while several wild pig populations around the world contributed to the domestic pig [10]. In contrast, domestic goat and sheep were domesticated only in one region [10].

We also found a relatively high divergence of the buffalo (swamp and river buffalo subspecies) on the molecular level, being at least as large as the divergence of taurine and zebu cattle [29]. We did not find any differences between river buffaloes from Italy and Sri Lanka, but identified three SNPs in a panel of 23 swamp buffaloes in Thailand, defining four haplotypes. These correlated clearly with the geographic origin of the animals. Haplotype YST2 has only been found once in Northern Thailand, while YST3 and YST4 were restricted to central Thailand [29]. Moreover, the presence of YST1 and YST4 in wild-domestic hybrids with Y-chromosomes from wild bulls implies a sharing of Y-chromosome haplotypes of wild and domestic populations. A more extensive sampling of domestic and wild populations near the Indian and Chinese domestication sites and in other Asian countries is required to more fully describe the pattern of Y-chromosomal variation. That may very well prove to be relevant for the genetic management of water buffalo population.

Analyzing maternal lineages, two mtDNA haplogroups A and B in the Chinese swamp buffalo population were found [14]. These were estimated to have diverged about 18000 ago, but do not have distinct geographical ranges. However, there was no comparison of the mtDNA diversity patterns of different countries, which precludes a more definite localization of the site of domestication site. In our study, we reported 63 mtDNA sequences of Thai swamp buffaloes from the northern, northeastern, middle and southern regions of Thailand. Our alignment showed that haplotypes were divided in two haplogroups (A and B lineages) as reported by [14]. The A and B lineages were represented by 19 and 11 haplotypes in the control regions and 8 and 5 haplotypes in the cytochrome b gene, respectively. Only four control region haplotypes [29] were shared by a sample of 119 Chinese swamp buffaloes [14]. The ratio of the A and B haplogroup did not clearly correlate with the geographical origin. This argues against a separate origin of the A and B haplogroup as reported by [14]. The haplotype diversity values revealed a diversity across Thailand that was higher than that calculated previously for the Chinese buffalo populations [14]. The observation of a haplotype intermediate between the A and B haplogroups confirms the high haplotype diversity in Thailand. Differences in the haplotype frequencies for the four populations [29] may partially reflect a limited sampling, but also suggest a differential geographic distribution of haplotypes. These observations do not support China as primary site of domestication and allow a tentative localization of the domestication site in Southeast Asia.

The swamp buffalo differs from European livestock species with regard to the genetic constitution. Swamp buffaloes lack obvious breed differentiation, but the mtDNA and Y-chromosomal haplotype patterns depend clearly on the geographic origin. This suggests a limited range of the gene flow via male or female migration. This may very well have preserved traces of the genetic history of the swamp buffalo and offers an interesting perspective for further characterization of the diversity and reconstruction of the history of the swamp buffalo. Such knowledge may enable the conservation of the diversity of available genes and contribute to the selection of genetically superior male and female buffaloes in order to improve the efficiency of buffalo breeding programs in the future.

Conclusions

The water buffalo is one of the most important domestic animals in Southeast Asia including Thailand. As the Thai swamp buffalo population declined during the last two decades, the swamp buffalo reproductive performance needs to be improved. Lack of knowledge on swamp buffalo reproduction, improper management and failure to use genetic superior males and females in breeding programs are the major factors to be considered. Artificial insemination was applied in Thailand but is inefficient due to suboptimal heat detection. Our study emphasized that the presence of a buffalo teaser bull is a very helpful tool for heat detection in the female swamp buffalo and thus for the proper insemination time either natural or artificial. There are also other signs of estrus of the female buffalo, which can be detected by trained experts. Postpartum anestrous is one of the major causes of poor reproductive performance in the buffalo leading to the long period of days open and thus a long calving interval. To improve postpartum buffalo reproduction efficiency, proper husbandry management such as quality of nutrition, optimal estrus detection and a fertile buffalo bull are crucial. In this study, the postpartum buffalo could be successfully mated within two months postpartum at the second spontaneous ovulation, even without hormonal treatment.

Embryo transfer would allow a wider exploitation and dissemination of superior swamp buffalo genotypes. We demonstrated that oocytes for IVM and IVF can be collected successfully by OPU without hormonal pretreatment, and those twice weekly pick-up results in better quality oocytes than once-weekly pick-up. In order to reinforce a more effective management of buffalo genetic resources and reconstruct their genetic history, we studied the genetic diversity of both paternal and maternal lineages. We found evidence for a separate domestication of the river and swamp buffalo, a shared wild-ancestral origin of the swamp buffalo A and B mtDNA haplotype and a Southeast Asian domestication site of the swamp buffalo. Occurrence of Y-chromosomal DNA variants depends on the region within Thailand, indicating a restricted migration of water buffaloes. Our data warrant a wider investigation of swamp buffalo mtDNA and Y-chromosomal diversity as well as a comprehensive study of autosomal variation. Insight in the genetic constitution of water buffalo may prove essential for the preservation and utilization of the genetic resources of Thailand.

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Appendix
Reproduction and genetic diversity of the swamp buffalo: Additional references [31] – [85]

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