Phylogenetic Position of Selembu and other Cattle via Paternal Marker Comparison

Md-Zain BM*, Majid S, Rosli MKA, Yaakop S

School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
*Corresponding author: abgbadd@ukm.edu.my, abgbadd1966@yahoo.com

Abstract: The Selembu (*Bos frontalis*) is an interspecific hybrid of the Gaur and domestic cattle. Here, we identified the phylogenetic position of the Selembu with other cattle via nuclear DNA sequence data using the Prolactin Receptor (PRLR). The genetic samples consisted of 25 cattle DNA samples from 5 Selembu, 4 Malayan Gaurs, 1 Banteng, 5 Bali, 4 Taurine and 6 Zebu cattle. DNA sequences of 890 bp were analyzed using Neighbor Joining (NJ) and Maximum Parsimony (MP). The tree topologies indicated that the Selembu deposited in a clade of domestic cattle consisting of Zebu and Taurine cattle. The PRLR gene showed its effectiveness in explaining the phylogenetic position of the Selembu with other cattle.

[Md-Zain BM, Majid S, Rosli MKA, Yaakop S. **Phylogenetic Position of Selembu and other Cattle via Paternal Marker Comparison.** *Life Sci J* 2014;11(12):42-46]. (ISSN:1097-8135). http://www.lifesciencesite.com. 8

Keywords: Selembu, Malayan gaur, *Bos frontalis, Bos gaurus*

1. Introduction

Cattle are classified as in the order Artiodactyla, family Bovidae, subfamily Bovinae and the genus *Bos* (Wilson and Reeder 2005). The genus is divided into two main groups: wild cattle and domestic cattle. Domestic cattle are bred for commercialization purposes, while wild cattle usually live in their natural habitats or are confined for conservation purposes. The Gayal (*Bos frontalis*) is a rare semi-wild bovine distributed throughout Bangladesh, Bhutan, China, India, Malaysia and Myanmar (Rajkhowa et al 2006). Due to the remoteness of their habitats, socio-political and ecological factors, the Gayal is one of the least studied ungulates.

In Malaysia, the Gayal is known as the Selembu based on the hybrid name composed of Seladang (gaur) and domestic cattle. According to Mamat-Hamidi et al. (2009) the hybrid was accidentally formed when a lost male Malayan Gaur entered a dairy farm owned by Malaysia Veterinary Services at Kluang, Johor. The bull mated with a female Heifer which delivered the first Selembu in Malaysia in April of 1983. These cattle have preeminent beef-type characteristics, but the male hybrid is usually infertile (Wilson and Reeder 2005). Crossbreeding with *Bos indicus* (Zebu cattle) is for adaptability, and crossbreeding with *Bos taurus* (Taurine cattle) is for productivity. However, due to the fertility problems of the male hybrids, the project of Selembu breeding was abandoned and the hybrids were kept with the dairy herd.

In most systematic studies, mtDNA has been extensively used to amplify genes of interest (Mastoor et al 2014; Abdul-Latiff et al 2014), because it has a high copy number and high mutation rate (Rosli et al 2014). Although the mutation rate of mtDNA is fairly high, it is reasonably fixed, making it quite useful to trace DNA changes through time. However, mtDNA is biased toward the maternal lineage, while nuclear DNA is inherited parentally, providing information from both the maternal and paternal lineages (Verkaar et al 2004). Thus, this study was conducted using molecular data from nuclear DNA, the PRLR gene, to infer the status of Selembu with regard to other cattle. PRLR was used in previous studies on Artiodactyla, including those of the American bison, European bison and Yak (Iso-Touru et al 2009). According to Haig (2008), PRLR has undergone greatly accelerated evolutionary change; however, this gene is evolutionarily conservative, with a slow rate of amino acid substitution.

2. Material and Methods

A total of 28 samples were used in this study (Table 1), where 27 of the samples represent the ingroup and one sample represents the outgroup. Ingroup taxa consist of 4 individuals of Seladang, 1 individual of Banteng (*B. javanicus birmanicus*), 5 individuals of Bali cattle, 5 individuals of Selembu, and 12 individuals of domestic cattle. The water buffalo (*Bubalus bubalis*) was used as an outgroup. QIAGEN DNeasy Blood & Tissue and QIAGEN QIAamp DNA Stool Mini Kits were used for the DNA extraction, and DNA amplification was performed using the standard polymerase chain reaction (PCR) and Master Mix reagents. The Prolactin Receptor Gene (PRLR) was selected as the gene fragment of interest and amplified by using a

Master Cycler machine (Eppendorf). PCR was performed in a 25 µL mixture containing ddH_2O, PCR buffer, dNTP, primers (forward AGA ACT TCT GCG AGC TCT GG and reverse GCC TTG GCT GGA TTC TAT GG), Taq polymerase and whole-genomic DNA as the template. The reaction volume and final concentrations of each PCR reagent used are further listed in Table 2, followed by the PCR cycle profile in Table 3. The PCR product was purified using the GF-1 PCR Clean-up Kit (Vivantis) and sent to First BASE Laboratories Sdn Bhd for DNA sequencing services.

Table 1 List of genetic samples

No	Species	Local Name	Sample Code
1.	*B. gaurus*	Seladang	Sarum
2.	*B. gaurus*	Seladang	BG 8
3.	*B. gaurus*	Seladang	Waja
4.	*B. gaurus*	Seladang	BG 17
5.	*B. javanicus*	Banteng	BJM 3
6.	*B. javanicus*	Bali	G
7.	*B. javanicus*	Bali	I
8.	*B. javanicus*	Bali	F
9.	*B. javanicus*	Bali	H
10.	*B. javanicus*	Bali	C
11.	*B. frontalis*	Selembu	S1
12.	*B. frontalis*	Selembu	S2
13.	*B. frontalis*	Selembu	S3
14.	*B. frontalis*	Selembu	S4
15.	*B. frontalis*	Selembu	S6
16.	*B. taurus*	Droughtmaster	Taurine J2
17.	*B. taurus*	Drankenberger	Taurine J4
18.	*B. taurus*	Limousin	Taurine J11
19.	*B. taurus*	Jersey	Taurine J13
20.	*B. indicus*	Kedah Kelantan	Zebu J6
21.	*B. indicus*	Fresian	Zebu J9
22.	*B. indicus*	Kedah-Kelantan	Zebu KK2
23.	*B. indicus*	Kedah-Kelantan	Zebu KK4
24.	*B. indicus*	Kedah-Kelantan	Zebu KK13
25.	*B. indicus*	Kedah-Kelantan	Zebu KK46
26.	*B. indicus*	Zebu cattle	FJ901285.1
27.	*B. taurus*	Taurine cattle	FJ901293.1
28.	*B. bubalis*	Water buffalo	NC006295.1

Table 2. PCR components

PCR Component	Optimal concentration	Volume (ul)
Distilled water (ddH_2O)	-	17.65
PCR buffer 10X	1X	2.5
dNTP mix (10 mM)	0.24 mM	0.5
$MgCl_2$ (50 mM)	2.0 mM	2.1
Forward primer (10 uM)	0.2 uM	0.5
Reverse primer (10 uM)	0.2 uM	0.5
Taq Polymerase (5U/ ul)	1 U	0.25
DNA template	50 ng/uL	1.0
Total	-	25.0

Table 3 PCR cycle profile

Parameter	Temperature (°C)	Time period
Initial Denaturation	94	4 minutes
Denaturation	94	30 sec
Annealing	62	30 sec
Elongation	72	1 minute
Final Elongation	72	7 minutes
Store	4	∞

The DNA sequence data were analyzed using MEGA version 4.0.2 and Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10. The phylogenic trees were constructed by using Neighbor Joining (NJ), Maximum Parsimony (MP) and Bayesian inference analyses. The NJ analysis was completed using the Kimura-2-parameter model for calculating genetic distances, while the MP analysis was performed with heuristic searches and 1000 random stepwise additions, with the application of a 50% of consensus majority rule. The phylogenetic tree was constructed using the Tree Bisection and Reconnection (TBR) method, and Bootstrap analysis was applied using 1000 replicates. The Bayesian inference analysis was done based on the optimum replacement model and phylogenetic parameter that fit the Akaike Information Criterion (AIC) in the Modeltest 3.7 software. The analysis was performed using two algorithms simultaneously: metropolis-coupled and Markov Chain Monte Carlo (MCMC) up to 1000000 generation, or no less than 0.01 divergence frequency.

3. Results and Discussion

Electrophoresis of the blood extraction samples showed a clear DNA band, while the fecal extraction samples showed smearing of the DNA band on agarose gel (Figure 1).

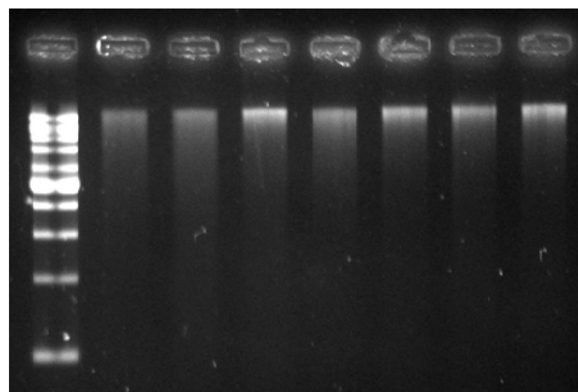

Fig. 1 DNA extraction of blood samples on 1.5% agarose gel.

Several trials on the annealing temperature were conducted in the range from 61ºC to 62ºC, and Figure 2 shows the DNA bands of the PCR products

at 61°C on 1.5% agarose gel. Temperatures at or above the *Tm* may ensure better specificity at the expense of sensitivity. All samples were successfully sequenced up to 900 bp, and aligned using BioEdit Software. The domestic cattle control sequences of the Zebu (FJ901285.1) and Taurine cattle (FJ901293.1) obtained from GenBank were aligned together with all DNA sequences studied, and some adjustments were made to obtain the same base pair lengths to construct the phylogenetic tree.

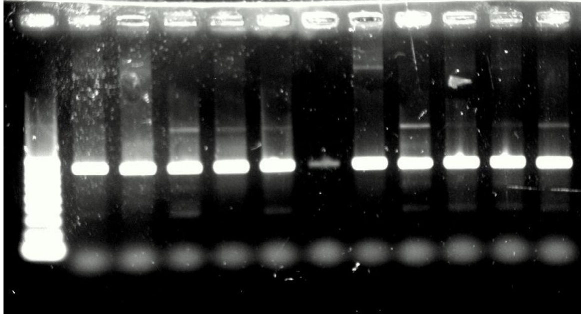

Fig. 2. Amplification products on 1.5% agarose gel

The sequence length used for the analyses was 824 base pairs, although the sequences amplified were approximately 900 base pairs. From the 824 bp analyzed, 773 (93.81%) were constant characters, 4.37% were parsimony non-informative and 1.82% of the characters were parsimony informative. The ratio of transition/transversion (Ti/Tv) of the sequences was 2.74 for purine and 4.64 for pyrimidine, with a bias value (R) of 2.03 (Table 4). The analysis of the genetic distance within the genus *Bos* was carried out by using Kimura's two-parameter (K2P) model, which includes both substitutions (transitions and transversions). Table 5 shows the genetic distance percentages between the Selembu and other species in the genus *Bos*. The genetic distances obtained were in the range of 0.1% to 2.6%. The smallest value for the genetic distance was 0.1%, between the Selembu and Taurine groups, while the largest value of genetic distance was 2.6%, between the Seladang and the outgroup species, water buffalo.

The Neighbor-Joining (NJ) phylogenetic tree analysis was generated using the Kimura-2-Parameter with 1000 bootstrap replication (Figure 3). All species of the genus *Bos* are clustered in the main clade that was separated from the outgroup species, and this clade was supported by a low bootstrap value. In Clade A, the species of genus *Bos* diverge into 2 different clades: Clade B, consisting of Bali cattle with a 40% bootstrap value, and Clade C, consisting of the Seladang, Taurine, Zebu, Selembu and Banteng, with a bootstrap value of 51%. Clade C is further divided into two clades, Clade D and Clade E: Clade D consists of the Seladang, Taurine and Zebu cattle, supported by a 40% bootstrap value, while Clade E groups the Selembu, Taurine, Zebu and Banteng cattle, supported by a 40% bootstrap value. The MP phylogenetic tree analysis with the best parsimony tree selected with attributes includes: consistency index (CI) = 0.9123, homoplasy index (HI) = 0.0877, retention index (RI) = 0.9333, rescaled consistency index (RC) = 0.8515 and tree length = 57. The same topologies produced by the NJ and MP revealed monophyly of the ingroup with respect to the outgroup, *Bubalus bubalis*. However, all bootstrap values in the MP tree were below 50% (Figure 4).

Table 4. Sequences analyses of the genus *Bos*

Analyses	Number/ Value
Total characters	824
Constant characters	773
Variable characters	51
Parsimony non-informative characters	36
Parsimony informative characters	15
Percentage of parsimony informative character (%)	1.82%
Ratio of Ti/Tv (purines)	2.74
Ratio of Ti/Tv (pyrimidines)	4.64
Bias Ti/Tv (R)	2.03
Variable characters after excluding outgroup	44
Parsimony non-informative characters after excluding outgroup	29
Ratio of Ti/Tv (purines) after excluding outgroup	2.68
Ratio of Ti/Tv (pyrimidines) after excluding outgroup	5.23
Bias Ti/Tv (R) after excluding outgroup	2.02
Nucleotide A frequencies	0.251
Nucleotide T frequencies	0.185
Nucleotide C frequencies	0.322
Nucleotide G frequencies	0.242

Table 5 Genetic distance in percentage (%)

	Bali	Banteng	Seladang	Selembu	Zebu	Taurine
Banteng	0.5					
Seladang	1.5	1.5				
Selembu	0.3	0.2	1.2			
Zebu	0.5	0.4	1.2	0.2		
Taurine	0.5	0.4	1.2	0.1	0.2	
Buffalo	1.7	2.1	2.6	1.8	1.8	1.8

The tree topologies from the different phylogenetic analyses indicated that Zebu, Taurine, Selembu and Banteng were grouped together in one major clade. The Zebu and Taurine cattle were domesticated from the same ancestor, the Aurochs

(*B. primigenius*), while introgression of the Selembu in that clade is because the Selembu is a hybrid of wild cattle (Seladang) and Zebu or Taurine domestic cattle. These results are supported by previous studies using mtDNA and the SRY of the Y-chromosome data (Guolong et al 2009; Syed-Shabthar et al 2013). However, several report findings have indicated that there are three deposition possibilities of this hybrid: a) Selembu will be deposited in an independent clade that has a close relationship with Seladang (Gaur) (Verkaar et al 2004), b) Selembu will be deposited in a clade together with Seladang (Gaur) (Ma et al 2007), and c) Selembu will be deposited in a clade with either Zebu or Taurine cattle (Syed-Shabthar et al 2013; Li et al 2008). This was also supported by a low value of genetic distance between the Selembu and the Zebu and Taurine cattle. Bali cattle form their own monophyletic clade. They were previously grouped in the wild cattle clade according to maternal inheritance (Rosli et al 2011), and formed their own monophyletic clade in a major clade that consists of domestic cattle, according paternal inheritance. The Seladang formed its own monophyletic clade in a major clade that consists of the Taurine and Zebu cattle.

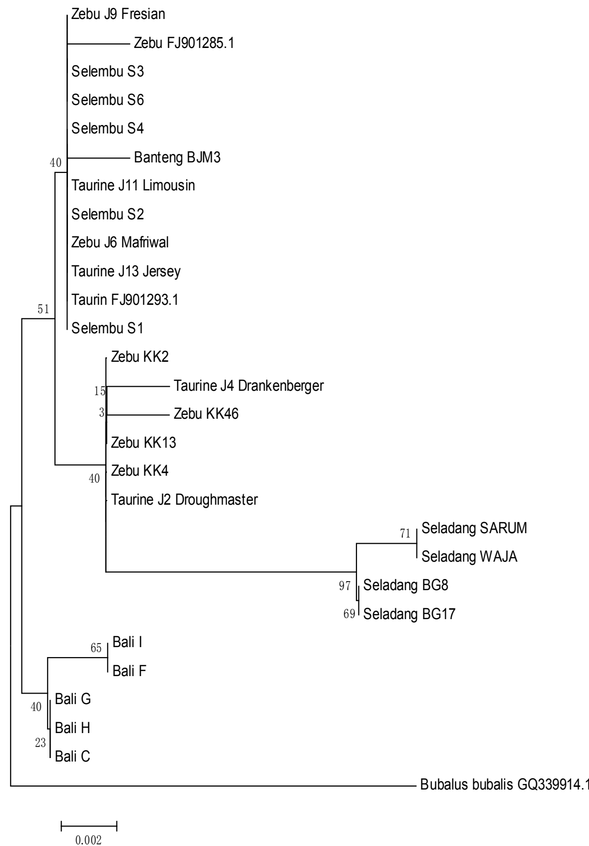

Fig. 3 Neigbour-joining tree

Differences in the mutation rate between species are essential to conclude a more significant grouping in tree topologies, as they provide more information on genetic differences. According to Haig (2008), the PRLR gene is evolutionarily conservative in most mammals, with a slow rate of amino acid substitution. In this study, the mutation rate of the PRLR gene was low; therefore, there is insufficient information for constructing a well-resolved phylogenetic tree. The low number of variable characters may be a disadvantage in conducting phylogenetic analyses because it reduces the possibility of detecting any nucleotide differences that would distinguish each species. In this study, the final length of the sequence that was used for analysis was shorter than the actual length of the amplified DNA fragments. Thus, cutting several base pairs may have reduced the percentage of variable characters, hence minimizing the effectiveness of the PRLR gene in resolving the relationships.

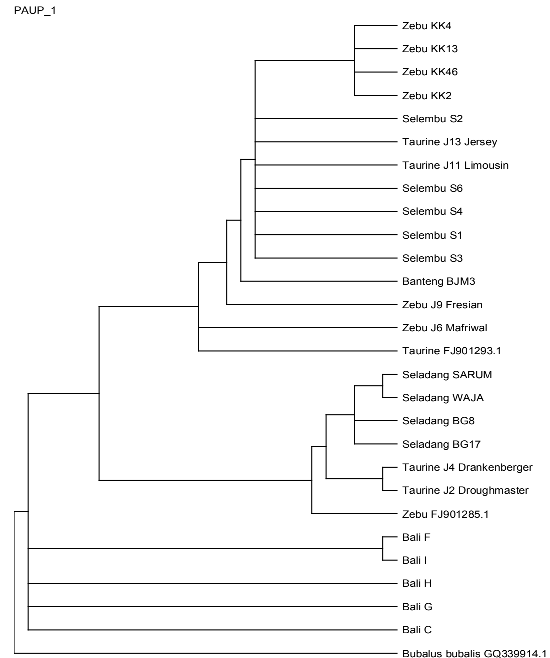

Fig. 4 Maximum parsimony tree

The phylogenetic tree that was constructed using the PRLR gene sequence data provided a weak resolution, due to the presence of polytomy and insertion of a Banteng individual in a clade consisting of domestic cattle (Zebu, Taurine and Selembu). Furthermore, the divergence of each clade was not supported by a high bootstrap value as seen in the maximum parsimony tree, compared to previous studies that employed mitochondrial DNA data.

In conclusion, the PRLR gene is less effective in resolving the relationship of cattle species because it has a low number of variable sites and a weak resolution of the phylogenetic tree. However, the PRLR gene showed some effectiveness in explaining the phylogenetic relationships of the Selembu with other cattle. The polyphyletic clade of the Selembu with domestic cattle (Zebu and Taurine cattle) resulting from this research showed that the Selembu, Zebu and Taurine cattle share a common ancestor. Therefore, referring to the PRLR gene alone is not sufficient for a thorough phylogenetic relationship analysis of species in the genus *Bos.*

Acknowledgements:

We would like to express our sincere appreciation to the Faculty of Science and Technology, UKM. We also thank the Agro-Biotechnology Institute (ABI), MOSTI, Department of Wildlife and National Parks (PERHILITAN), and the National Institute of Veterinary Biodiversity (IBVK) for providing genetic samples. This study was funded by research grants STGL-003-2009, INDUSTRI-2011-003, 08-05-ABI-AB032/1, Sciencefund 02-01-02-SF0762, and DLP-2013-006.

Corresponding Author:
Dr. Badrul Munir Md-Zain
School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
E-mail: abgbadd@ukm.edu.my

References

1. M.A.B. Abdul-Latiff, F. Ruslin, V.F. Vun, A. Mohd-Hashim, J.J. Rovie-Ryan, P. Abdul-Patah, M. Lakim, C. Roos, S. Yaakop, and B.M. Md-Zain, “Phylogenetic relationships of Malaysia’s long-tailed macaques, *Macaca fascicularis*, based on cytochrome *b* sequences,” *ZooKeys* vol. 407, pp.121-139, 2014.
2. M. Guolong, C. Hong, L. Shiping, C. Hongyu, J. Dejun, G. Rongqing, C. Chunfang, and L. Yonghong, “Phylogenetic Relationships and Status Quo of Colonies for Gayal Based on Analysis of Cytochrome *b* Gene Partial Sequences,” *Journal of Genetics and Genomics,* vol. 34, pp. 413-419, 2007.
3. D. Haig, “Placental growth hormone-related proteins and prolactin-related proteins,” *Trophoblast Research,* vol. 22, pp. 36-41, 2008.
4. T. Iso-Touru, J. Kantanen, L. Meng-Hua, Z. Gizejewski, and J. Vilkki, “Divergent evolution in the cytoplasmic domains of PRLR and GHR genes in Artiodactyla,” BMC Evolution Biology, vol. 9: pp. 172, 2009.
5. S.P. Li, H. Chang, G.L Ma, and H.Y. Chang, “Molecular phylogeny of the gayal in Yunnan China inferred from the analysis of cytochrome b gene entire sequences,” *Asian Austral Journal of Animal,* vol. 21, pp. 789-793, 2008.
6. G. Ma, H. Chang, S. Li, H. Chen,D. Ji, R, Geng, C. Chang, and Y. Li, “Phylogenetic relationship and status quo of colonies for gayal based on analysis of Cytochrome b gene partial sequences,” *Journal of Genetic Genome,* vol. 34, pp. 413-419, 2007.
7. Mamat-Hamidi, I. Idris, and M. Hilmi, “Karyotype of Malayan gaur (Bos gaurus hubbacki), shahiwal-fresian cattle and gaur x cattle hybrid backcrosses,” *Pakistan Journal of Biological Sciences,* vol. 12, pp. 896-901, 2009.
8. N.H. Masstor, A. Samat, M.N. Shukor, and B.M. Md-Zain, “Molecular Phylogeny of the Bamboo Sharks (*Chiloscyllium* spp.),” *BioMed Research International*, vol. 2014, Article ID 213896, 2014.
9. S. Rajkhowa, D.K. Sarma, and C. Rajkhowa, “Seroprevalence of *Toxoplasma gondii* antibodies in captive mithuns (*Bos frontalis*) from India,” *Veterinary Parasitol*ogy, vol. 135, pp. 369-374, 2006.
10. M.K.A. Rosli, S.S. Zakaria, S.M.F. Syed-Shabthar, Z.Z. Zainal, M.N. Shukor M.C. Mahani, O. Abas-Mazni, and B.M. Md-Zain, “Phylogenetic relationships of Malayan gaur with other species of the genus Bos based on cytochrome b gene DNA sequences,”*Genetics and Molecular Research,* vol. 10, pp. 482-93, 2011.
11. M.K.A. Rosli, S.M.F. Syed-Shabthar, P. Abdul-Patah, Z. Abdul-Samad, S.N. Abdul, M.N. Burhanuddin, N.A. Zulkifli, M.N. Shukor, K. Budsabong, S. Changtragoon, T. Sekiguchi, H. Sasaki, and B.M. Md-Zain, “A New Subspecies Identification and Population Study of theAsian Small-Clawed Otter (*Aonyx cinereus*) in Malay Peninsula and Southern Thailand Based on Fecal DNA Method,” *The ScientificWorld Journal*, vol. 2014, Article ID 457350, 2014.
12. S.M.F. Syed-Shabthar, M.K.A. Rosli, S.M.N Romaino, Z.A. Fazly-Ann, M.C. Mahani, O. Abas-Mazni, R. Zainuddin, Yaakop, S. and B.M. Md-Zain, “The molecular phylogenetic signature of Bali cattle revealed by maternal and paternal markers,” *Molecular Biology Reports,* vol. 40, pp. 5165-5176, 2013.
13. E.L.C. Verkaar, I.J. Nijman, M. Beeke, E. Hanekamp, and J.A. Lenstra, “Maternal and paternal lineages in cross-breeding bovine species. Has wisent a hybrid origin?,” *Molecular Biology and Evolution,* vol. 21(7): pp. 1165-1170, 2004.
14. D.E. Wilson, and D.A.M. Reeder, *Mammal Species of the world. A taxonomic and geographical reference.* Baltimore: John Hopkins University Press, 2005.

7/11/2014