Systematic relationships of five newly sequenced cervid species

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ABSTRACT

Cervid phylogenetics has been puzzling researchers for over 150 years. In recent decades, molecular systematics has provided new input for both the support and revision of the previous results from comparative anatomy but has led to only partial consensus. Despite all of the efforts to reach taxon-wide species sampling over the last two decades, a number of cervid species still lack molecular data because they are difficult to access in the wild. By extracting ancient DNA from museum specimens, in this study, we obtained partial mitochondrial cytochrome b gene sequences for \textit{Mazama bricenii}, \textit{Mazama chunyi}, \textit{Muntiacus atherodes}, \textit{Pudu mephistophiles}, and \textit{Rusa marianna}, including three holotypes. These new sequences were used to enrich the existing mitochondrial DNA alignments and yielded the most taxonomically complete data set for cervids to date. Phylogenetic analyses provide new insights into the evolutionary history of these five species. However, systematic uncertainties within \textit{Muntiacus} persist and resolving phylogenetic relationships within \textit{Pudu} and \textit{Mazama} remain challenging.

INTRODUCTION

Cervidae forms a subclade of ruminant artiodactyls and is the second most diverse group among terrestrial artiodactyls, with 55 extant species (\textit{IUCN, 2015}), including one recently extinct species (\textit{Rucervus schomburgki}; \textit{Duckworth, Robichaud & Timmins, 2008}). Cervids natively inhabit Eurasia, the Americas, and potentially northernmost Africa (\textit{Mattioli, 2011}). They are adapted to diverse climatic zones, ranging from the tropics to arctic regions, and to diverse habitats such as tundra, grasslands, swamps, forests, woodlands, and ecotones (\textit{Mattioli, 2011}). Their unique phenotypic feature is a pair of antlers, which are osseous outgrowths of the frontal bone that are shed and rebuilt regularly. The current conservation status of cervids lists 29 species as ‘threatened’, nine species as ‘data deficient’, and 17 species as ‘least concern’ in the IUCN Red List of Threatened Species (\textit{IUCN, 2015}). Samples and life history data are much more difficult to obtain from rare and threatened species than from more abundant species. Therefore, there is a discrepancy between the well-studied (e.g., \textit{Cervus elaphus}, red deer; \textit{Odocoileus hemionus}, mule deer; \textit{Rangifer...
Figure 1  Overview of the current state of the art of cervid classification based on literature (e.g., Hassanin & Douzery, 2003; Pitra et al., 2004; Kuznetsova, Kholodova & Danilkin, 2005; Hernández-Fernández & Vrba, 2005; Hughes et al., 2006; Gilbert, Ropiquet & Hassanin, 2006; Marcot, 2007; Agnarsson & May-Collado, 2008; Duarte, González & Maldonado, 2008; Hassanin et al., 2012). The diagram shows the different clades, their geographical origination, and their current distribution.

tarandus, reindeer) and barely known species (e.g., Mazama spp., brocket deer; Pudu spp., pudu; Muntiacus spp., muntjac). Consequently, data for the latter taxa are overdue.

Cervid phylogenetics has improved considerably in recent decades through molecular systematics (e.g., Hassanin & Douzery, 2003; Pitra et al., 2004; Kuznetsova, Kholodova & Danilkin, 2005; Hernández-Fernández & Vrba, 2005; Hughes et al., 2006; Gilbert, Ropiquet & Hassanin, 2006; Marcot, 2007; Agnarsson & May-Collado, 2008; Duarte, González & Maldonado, 2008; Hassanin et al., 2012). However, several species are still underrepresented in molecular phylogenetic analyses because their current conservation status of threatened or data deficient negatively affects their sample collection.

Consensus has been reached for the monophyly of taxa Cervidae, Muntiacini, Cervini, Capreolini and Odocoileini. Muntiacini and Cervini form the clade Cervinae, which is a sister taxon to Capreolinae comprising Odocoileini, Rangiferini, Capreolini and Alceini (e.g., Hernández-Fernández & Vrba, 2005; Gilbert, Ropiquet & Hassanin, 2006; Hassanin et al., 2012). The Capreolinae-Cervinae-split is commonly supported in previously published topologies and corresponds to the first (though not formally valid) morphological cervid classification by Brooke (1878), who differentiated Plesiometacarpi and Telemetacarpi (Fig. 1). Systematic relationships within Cervinae appear to be largely resolved, whereas Capreolinae systematics is more controversial (Pitra et al., 2004; Gilbert, Ropiquet & Hassanin, 2006; Duarte, González & Maldonado, 2008; Hassanin et al., 2012; Croitor, 2014). For an overview of current cervid classifications, see Fig. 1.
The mitochondrial cytochrome b (Cytb) gene is the best-sampled across cervids. Cytb is a marker that is known to be highly variable in mammals, which makes it a suitable marker for resolving genus and species level relationships but less suitable for resolving deeper nodes (family level and above) or for population studies (Hofreiter et al., 2001a). In addition, because mitochondrial genomes are maternally inherited, they may not allow a full reconstruction of a species’ evolutionary history if there is no random mating.

However, Hassanin et al. (2012) sequenced and analysed mitochondrial genomes of 33 cervid species as part of a large Artiodactyla phylogenetic reconstruction and provided a robust phylogenetic framework for cervids. To date, sampling of mitochondrial genomes and individual partial Cytb sequences cover 46 of the 55 cervid species. Here, we present the results of phylogenetic analyses that include four species not previously sampled for molecular data: Mazama chunyi (Peruvian dwarf brocket), Muntiacus atherodes (Bornean yellow muntjac; including holotype), Pudu mephistophiles (Northern pudu; including holotype), and Rusa marianna (Philippine brown deer), all of which were taken from museum specimens. We also sequenced three Mazama bricenii museum specimens (Mérida brocket; including the holotype), of which Cytb sequences have been published recently and were sequenced contemporaneously with our study (Gutiérrez et al., 2015). Except for M. atherodes (least concern), all species have been assessed as vulnerable based on the IUCN Red List. Therefore, considering the threat of extinction, our approach of sequencing DNA from museum material is an important contribution to cervid systematics.

The specific aims of our study were (1) to reconstruct the systematic position of M. bricenii and M. chunyi and further investigate the polyphyly of the genus Mazama, (2) to reconstruct the systematic position of M. atherodes, (3) to test the monophyly of the Philippine Rusa species (R. alfredi and R. marianna) and their sister taxon position relative to the Indonesian and mainland Rusa species (R. timorensis and R. unicolor), and (4) to test the monophyly of Pudu.

To achieve these aims, we experimented with different matrix sizes and parameters to examine the reliability of the phylogenetic signal throughout different data sets.

**MATERIAL & METHODS**

**Material**

We sampled and sequenced five species from which no molecular data were available previous to our study (but see Gutiérrez et al., 2015) (Tables 1 and 2). Samples were taken from thirteen museum specimens, nine from the Natural History Museum in London (BMNH) and four from the Museum für Naturkunde Berlin (ZMB). Three specimens represent holotypes (BMNH 1908.6.24.5 Mazama bricenii, BMNH 1971.3088 Muntiacus atherodes, BMNH 1896.1.28.6 Pudu mephistophiles). One sample was derived from a wet specimen, one from a skin, and the remaining samples consisted of bone fragments or dried soft tissue remains of skulls (details in Table 2). Figure 2 shows where the specimens originated and their currently known species distributions. The collection dates of each specimen are given in Table 2.
We obtained complete Cytb and/or mitochondrial genome sequences from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for 48 cervid species. These included the 45 extant cervids (full set of available extant cervid data excluding recently published M. bricenii sequences; Gutiérrez et al., 2015), one subspecies (Cervus elaphus canadensis), a questionable P. mephistophiles sequence from Hassanin et al. (2012), and one fossil cervid species (Megaloceros giganteus). We also added six non-cervid ruminant taxa (Table 1). The resulting Cytb data set is the most taxonomically extensive for Cervidae to date.

**Extraction**

The challenges of sequencing ancient DNA are related to the degradation of DNA after an organism’s death triggered by exogenous processes such as oxidation and background radiation. These processes affect the sugar-phosphate backbone and nitrous bases of the DNA strand, whereas hydrolytic processes such as depurination and deamination cause...
| Species                               | Cytochrome b | mtGenome |
|---------------------------------------|--------------|----------|
| Alces alces                           | AJ000026     | JN632595 |
| Alces americanus                      | M98484       | –        |
| Axis axis                             | AY607040     | JN632599 |
| Axis kuhlii                           | HQ893538     | –        |
| Axis porcinus                         | DQ379301     | JN632600 |
| Blastocerus dichotomus                | JN632603     | –        |
| Capreolus capreolus                   | AJ000024     | JN632610 |
| Capreolus pygargus                    | AJ000025     | –        |
| Cervus albirostris                    | AY044863     | JN632690 |
| Cervus elaphus canadensis             | AF423198     | –        |
| Cervus elaphus                        | JF489133     | NC_007704|
| Cervus nippon                         | JF893484     | NC_006993|
| Dama dama                             | AJ000022     | JN632629 |
| Dama mesopotamica                     | AY607034     | JN632630 |
| Elaphodus cephalophus                 | NC_008749    | NC_008749|
| Elaphurus davidianus                  | AF423194     | JN632632 |
| Hippocamelus antisensis 1             | JN632646     | NC_007704|
| Hippocamelus antisensis 2             | JN632646     | NC_007704|
| Hippocamelus bisulcus                 | DQ789177     | –        |
| Hydropotes inermis                    | AJ000028     | JN632649 |
| Mazama americana 1                    | DQ789209     | JN632656 |
| Mazama americana 2                    | DQ789217     | –        |
| Mazama americana 3                    | JN632657     | –        |
| Mazama americana 4                    | DQ789221     | –        |
| Mazama americana 5                    | DQ789219     | –        |
| Mazama bororo                          | DQ789187     | –        |
|                                        | DQ789231     | –        |
|                                        | DQ789228     | –        |

(continued on next page)
| Species                  | Cytochrome b          | mtGenome   |
|-------------------------|-----------------------|------------|
| **Mazama bricenii**     | LT546656              | –          |
|                         | LT546657              |            |
|                         | LT546658              |            |
| **Mazama chunyi**       | LT546655              | –          |
| **Mazama gouazoubira 1**| JN632658              | JN632658   |
|                         | NC_020720             |            |
|                         | DQ379308              |            |
| **Mazama gouazoubira 2**|                       |            |
| **Mazama nana**         | DQ789210              | –          |
|                         | DQ789214              |            |
|                         | DQ789227              |            |
| **Mazama nemorivaga 1** | JN632660              | JN632660   |
| **Mazama nemorivaga 2** | DQ789205              | JN632659   |
|                         | DQ789206              | NC_024812  |
|                         | DQ789226              |            |
| **Mazama pandora**      | KC146954              | –          |
|                         | KC146955              |            |
| **Mazama rufina**       | JN632661              | JN632661   |
|                         | NC_020721             |            |
| **Mazama temama**       | KC146956              | –          |
|                         | KC146957              |            |
|                         | KC146958              |            |
|                         | KC146959              |            |
| † *Megaloceros giganteus*| AM182644              | –          |
|                         | AM182645              |            |
| **Muntiacus atherodes** | LT546659              | –          |
| **Muntiacus crinifrons**| NC_004577             | NC_004577  |
|                         | AY239042              |            |
|                         | DQ445734              |            |
|                         | DQ445732              |            |
|                         | DQ445735              |            |
|                         | DQ445733              |            |
| **Muntiacus feae**      | AF042721              | –          |
| **Muntiacus muntjak 1** | NC_004563             | NC_004563  |
|                         | AY225986              |            |
| **Muntiacus muntjak 2** | AI042718              |            |
| **Muntiacus putaoensis**| EF523665              | –          |
|                         | EF523666              |            |
|                         | EF523667              |            |
|                         | EF523668              |            |
|                         | EF523669              |            |

(continued on next page)
| Species                      | Cytochrome b          | mtGenome    |
|------------------------------|-----------------------|-------------|
| *Muntiacus reevesi*          | AF327537              | NC_004069   |
| *Muntiacus rooseveltorum*    | KJ425278, KJ425279,   |             |
|                              | KJ425281, KJ425282    |             |
| *Muntiacus truongsonensis 1* | KJ425277              |             |
| *Muntiacus truongsonensis 2* | KJ425276              |             |
| *Muntiacus vuquangensis*     | FJ705435, AF042720    | FJ705435    |
| *Odocoileus hemionus 1*      | HM222707              | JN632670    |
| *Odocoileus hemionus 2*      | FJ188783, FJ188870    |             |
| *Odocoileus virginianus 1*   | DQ379370, M98491      | JN632671    |
| *Odocoileus virginianus 2*   | DQ789190, DQ789193    | JN632681    |
|                              | DQ789195, DQ789199    |             |
| *Pudu mephistophiles*        | JN632691, LT546651,   |             |
|                              | LT546652, LT546653,   |             |
|                              | LT546654              |             |
| *Pudu puda*                  | JN632692, AY607039,   | JN632692    |
|                              | NC_020740             |             |
| *Rangifer tarandus*          | AB245426, AY726704,   | NC_007703   |
|                              | KMS06758              |             |
| *Rucervus duvaucelii*        | AY607041              | JN632696    |
| *Rucervus eldii*             | AY157735              | JN632697    |
| *Rucervus schomburgki*       | AY607036              |             |
| *Rusa alfredi*               | JN632698, NC_020744   | JN632698    |
| *Rusa marianna*              | LT546647              |             |
|                              | LT546648, LT546649    |             |
|                              | LT546650              |             |
| *Rusa timorensis*            | AF423200              | JN632699    |
| *Rusa unicolor*              | FJ556575              | NC_008414   |

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Table 1 (continued)

| Species                  | Cytochrome b | mtGenome |
|--------------------------|--------------|----------|
| Antilocapra americana    | JN632597     | JN632597 |
| Boselaphus tragocamelus  | EF536350     | EF536350 |
| Hyemoschus aquaticus     | JN632650     | JN632650 |
| Moschus moschiferus      | FJ469675     | JN632662 |
| Okapia johnstoni         | JN632674     | JN632674 |
| Tragelaphus scriptus     | AF022067     | JN632706 |

breakage in the DNA molecules (Hofreiter et al., 2001b). Due to the large number of mitochondria per cell, mitochondrial gene sequences are more likely to be retrieved from ancient material than is nuclear DNA (Hofreiter et al., 2001a).

DNA was extracted using the Qiagen QIAamp DNA Micro Kit, including an overnight lysis step, following the manufacturer’s protocol. After lysis, 1 µg dissolved carrier RNA was added, as recommended in the protocol, 80 µl elution buffer was used for the last elusion step, and the last incubation step was set for five minutes instead of one minute. After the extraction, the DNA concentration was measured using a spectrometer (NanoDrop 1000; Peqlab Biotechnologie GmbH, software version ND 1000 v3.7.1) (Table 2).

**PCR**

Eight cervid-specific Cytb primers (Lister et al., 2005) were used to amplify a 747 base pair region from the 1140-base-pair-long mitochondrial Cytb, from nucleotide position 64 to 810. Each primer pair amplified a 100–140-base-pair-long sequence with overlap to adjacent sequences (Lister et al., 2005; Table 2).

Polymerase chain reactions (PCR) were carried out using a TProfessional thermocycler (Biometra). Sequences amplified from each primer pair were validated against contamination with a negative control. The specific PCR components are given in Table 3. The PCR programme was as follows: initial denaturation at 95 °C for three minutes, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and a final extension at 72 °C for five minutes. Amplification of target sequences was initially attempted using the components in Table 3, column (a) and an annealing temperature of 55 °C. Some primer-sample combinations did not result in amplification products. Therefore, we experimented with the components, e.g., not adding Bovine Serum Albumin (BSA), changing the overall reaction volume, and/or increasing the concentration of magnesium chloride (Table 3). We also experimented with annealing temperatures ranging from 48 °C to 52 °C. These optimisations were successful in most cases; however, a few sections of the individual sequences for certain specimens could not be successfully amplified, which left gaps in the Cytb sequence (Table 2).

Successfully amplified PCR products were sequenced in both directions using the amplification primers and the ABI BigDyeTerminator 3.1 chemistry following the manufacturer’s protocol on a capillary sequencer (ABI 3730; AppliedBiosystems) in the Genomic Sequencing Unit, Faculty of Biology, LMU. After quality control, the approximately 100–140-base-pair-long forward and reverse sequencing reads were assembled into contigs.
Table 2  Overview of sampled specimens. Specimens in bold are holotypes. The category ‘sample DNA’ provides the weight of the tissue sample in the tube prior to DNA extraction and DNA concentration after extraction.

| Species                  | Collection ID | Accession no. | Sample (mg) | DNA (ng/µl) | Gaps in alignment | Collection entry | Locality                  | Material                                |
|--------------------------|---------------|---------------|-------------|-------------|-------------------|------------------|---------------------------|-----------------------------------------|
| Rusa marianna            | BMNH 1996.2   | LT546647      | 15.5        | 93.65       | –                 | 1996             | Philippines              | Soft tissue fragments*                  |
| Rusa marianna            | ZMB-MAM-75158 | LT546648      | 15.1        | 60.97       | –                 | NA               | Philippines, Luzon       | Soft tissue & bone fragments*            |
| Rusa marianna            | ZMB-MAM-20409 | LT546649      | 12.0        | 49.64       | –                 | 1915             | Captive animal           | Soft tissue & bone fragments*            |
| Rusa marianna            | ZMB-MAM-75146 | LT546650      | 26.2        | 38.67       | 403–467           | 1905             | US, Northern Mariana Islands | Soft tissue & bone fragments*            |
| Pudu mephistophiles      | BMNH 1899.2.18.20 | LT546651 | 30.5        | 97.35       | 64–118, 176–211   | 1899             | Ecuador                  | Soft tissue & bone fragments* ; juvenile |
| Pudu mephistophiles      | BMNH 1896.1.28.6 | LT546652  | 7.6         | 56.57       | –                 | 1896             | Ecuador, Paramo of Papallacta | Snippet of skin, including hair; immature |
| Pudu mephistophiles      | BMNH 1899.2.18.21 | LT546653 | 9.9         | 27.34       | 604–674, 784–810  | 1899             | Ecuador                  | Soft tissue & bone fragments* ; juvenile |
| Pudu mephistophiles      | ZMB-MAM-61577  | LT546654      | 165.8       | 325.57      | –                 | 1970             | Captive animal           | Wet specimen; neonatal                   |
| Mazama chunyi            | BMNH 1967.1362 | LT546655      | 15.6        | 56.22       | –                 | 1967             | Peru, Chiquis            | Soft tissue & bone fragments*            |
| Mazama bricenii          | BMNH 1913.4.24.3 | LT546656 | 36.0        | 74.17       | –                 | 1913             | Venezuela, Merida        | Soft tissue & bone fragments*            |
| Mazama bricenii          | BMNH 1908.6.24.5 | LT546657 | 2.4         | 7.07        | 288–394, 604–674  | 1908             | Venezuela                | Soft tissue & bone fragments*            |
| Mazama bricenii          | BMNH 1934.9.10.228 | LT546658 | 10.2        | 77.08       | –                 | 1934             | Ecuador, Pichincha       | Soft tissue & bone fragments*            |
| Muntiacus atherodes      | BMNH 1971.3088 | LT546659      | 23.3        | 87.60       | –                 | 1971             | Borneo, Brunei/Indonesia/Malaysia | Soft tissue & bone fragments*            |

Notes. BMNH, British Museum of Natural History London; ZMB, Zoological collections of the Museum für Naturkunde Berlin. *From skull. **From skull & mandible. & From mandible.
These individual contigs were then assembled into a contig with a maximum length of 747 base pairs using CodonCodeAligner v.3.7.1.1.

To ensure that a genuine cervid Cytb fragment has been amplified, the forward and reverse pre-assembly sequences from each primer, the individual contigs of forward and reverse strands and the final 747-base-pair-long contigs were each BLASTed against NCBI GenBank entries. Only fragments returning a cervid in the first 50 BLAST search results were used. In almost all cases, where the BLAST result was different from the cervid result, the sequences were found to be most similar to Bos taurus. This contamination is possibly caused by the BSA added to enhance PCR outcomes. Sequences were submitted to the European Nucleotide Archive under accession numbers LT546647–LT546659 (Tables 1 and 2).

### Alignment

The concatenated consensus sequences of each specimen were added to the existing Cytb data set (NCBI GenBank) and pairwise aligned by eye using Mesquite v.2.75 (Maddison & Maddison, 2011) and Seaview 4.2 (Guindon, Gascuel, & Guindon, 2010). The alignment was carefully checked for stop codons within the alignment and/or unusual nucleotide positions by translation into amino acids to ensure the absence of pseudogenes and sequencing errors. The IUPAC ambiguity code was used in few cases where character states could not be assessed unambiguously after a re-investigation of the raw sequence data. These ambiguities most likely represent misreads from the chromatogram due to the somewhat poor condition of the DNA. Because these ambiguous sites are not numerous, their impact on the phylogenetic signal is negligible.

In total, three different alignments were created. First, we aligned the new 747 base pair long sequences with the complete Cytb sequences from GenBank to form a data set of 1140 base pairs. The final data set contained 130 taxa (124 cervids, six other ruminants). Second,
to test whether the newly sequenced, shorter fragments carried a sufficient phylogenetic
signal, two further alignments were created. One alignment was exactly 747 base pairs
long, which was the same length as the new sequences, including internal gaps. The other
alignment excluded even the internal gaps and was 569 base pairs long. We also re-analysed
the cervid subset (33 species) of the complete mitochondrial genome alignment available
for Artiodactyla in Hassanin et al. (2012) without the new sequences. The taxon sampling
contained 39 cervid taxa and seven non-cervid ruminants.

**Phylogenetic analyses**

To test for the impact of alignment length on phylogenetic signal, we developed three align-
ments with varying base pair lengths. For each alignment, we used PartitionFinder (Lanfear
et al., 2012) to identify the optimal partitioning scheme and mutation model (Table 4).

A summary of all analyses undertaken including the models and partitioning scheme,
is shown in Table 4. PartitionFinder analysis on the 1140 Cytb data set resulted in a
scheme with three different partitions for the individual codon positions using SYM for
position 1, HKY for position 2, and GTR for position 3 for Bayesian inference analyses with
MrBayes v.3.2.4 (Ronquist et al., 2012) (in the following referred to as BI-1140-part). For the
maximum likelihood analyses with RAxML (Stamatakis, 2006), PartitionFinder suggested
GTR for all codon positions (ML-1140). Alternatively, we undertook a Bayesian inference
analysis without partitioning using the GTR model on the 1140-base-pair-long alignment
(BI-1140-unpart). We also undertook a Bayesian analysis with the Cytb alignment reduced
to 747 base pairs (BI-747-part) using the partitioning scheme suggested by PartitionFinder
and the models described above as well as one unpartitioned analysis (BI-747-unpart) using
GTR. Further, we undertook another Bayesian analysis on the 569 base pair alignment
(BI-569-unpart), excluding the internal gaps, representing the shortest sequence length
of the newly sequenced taxa (Maz_bri_Q_BMNH_1908.6.24.5). This analysis was run
using the GTR model and no partitioning because of the short alignment length. The
Bayesian re-analysis of the complete mitochondrial genome sequences (BI-mtG; without
the newly sequenced Cytb sequences) was undertaken using GTR and divided the data
set into seven partitions (Hassanin et al., 2012). The re-analysis was carried out because
previous re-analyses of subsets of the complete mitochondrial genome resulted in different
results than those found by Hassanin et al. (2012).

Substitution models for all analyses were implemented with a gamma distribution
(Γ) without a proportion of invariant sites (I), although PartitionFinder suggested using
Γ + I for most partitions. It is known that the combination Γ + I may create two areas of
equal probability in the tree landscape, which can lead to convergence problems (Moyle
et al., 2012). All Bayesian Inference analyses were run with MrBayes v.3.2.4 (Ronquist
et al., 2012) using Metropolis-Coupled Markov Chain Monte Carlo (MC³); two separate
runs sampled the tree landscape at a temperature of 0.35 sampling every 1,000th tree.
The mitochondrial genome analysis was run with MrBayes v.3.2.4 (Ronquist et al., 2012)
using MC³ with two separate runs sampling every 5,000th tree at a temperature of 0.35.
All analyses automatically stopped when the standard deviation of split frequencies
of posterior probabilities reached 0.01. From all post burn-in sampled trees, a consensus tree
Table 4  Summary of analyses, model choice, partitioning, and support for major clades in the resulting topologies.

| Analysis      | Reference    | Model(s) | Partitioned | Cervidae | Cervinae | Cervini | Muntiacini | Capreolinae | Capreolini | Odocoileini | Blastocerina | Odocoileina |
|---------------|--------------|----------|-------------|----------|----------|---------|------------|-------------|------------|-------------|--------------|-------------|
| BI-mtG        | Fig. 3A, Fig. S1 | GTR      | Y           | 1        | 1        | 1       | 1          | 1           | 1          | 1           | 1            | 1           |
| BI-1140-unpart| Fig. 3B, Fig. 4, Fig. S2 | GTR      | N           | 1        | 1        | 1       | 1          | –           | 1          | .84         | .98          | .99         |
|               |              |          | SYM         |          |          |         |            |             |            |             |              |             |
|               |              |          | HKY         |          |          |         |            |             |            |             |              |             |
|               |              |          | GTR         |          |          |         |            |             |            |             |              |             |
| BI-1140-part  | Fig. 3C, Fig. S3 | SYM      | Y           | 1        | .99      | 1       | 1          | –           | 1          | –           | .75          | .87         |
| ML-1140       | Fig. 3D, Fig. S4 | GTR      | N           | 99       | 89       | 99      | 92         | –           | 100        | 57          | 55           | 41          |
| BI-747-unpart | Fig. 3E, Fig. S5 | GTR      | N           | 1        | 1        | 1       | .81        | –           | 1          | –           | .85          | –           |
| BI-747-part   | Fig. 3F, Fig. S6 | SYM      | Y           | 1        | .99      | 1       | .90        | –           | 1          | –           | –            | –           |
|               |              |          | HKY         |          |          |         |            |             |            |             |              |             |
|               |              |          | GTR         |          |          |         |            |             |            |             |              |             |
| BI-569-unpart | Fig. 3G, Fig. S7 | GTR      | N           | 1        | –        | .99     | .92        | –           | 1          | –           | –            | –           |

Notes.

Abbreviations: BI, Bayesian Inference; ML, Maximum Likelihood, the number represents the Cytb sequence length in the current alignment; Y, yes; N, no; part, partitioned; unpart, unpartitioned. The values within cells represent the node support for the respective split either as Bayesian posterior probabilities or as bootstrap support from maximum likelihood analyses; “–” indicates that the clade was not recovered in the respective analysis.
Figure 3  Overview of higher level topologies resulting from re-analysis of the complete mitochondrial genome sequences (Hassanin et al., 2012) and six different analyses of our data set. (A) BI-mtG, (B) BI-1140-unpart, (C) BI-1140-part (D) ML-1140, (E) BI-747-unpart, (F) BI-747-part, (G) BI-569-unpart. Support values represent bootstrap values in D, all other support values are posterior probabilities. (A–D) show monophyly for all major cervid lineages, whereas in (E–G) resolution, particularly within Odocoileini is lost. Positioning P. mephistophiles proves to be difficult. Scale bars represent substitutions per site.

was generated (burn-in = 25%). For the Maximum Likelihood analysis we used RAxML v.7.3.0 (Stamatakis, 2006) including a rapid bootstrap search with 100 replicates on the 1140 base pair long data set.

Hyemoschus aquaticus (Tragulidae, Artiodactyla), which is an extant representative of crown ruminants, was used as the outgroup. The original tree topologies from all seven analyses are provided in Figs. S1–S7, and an overview is given in Fig. 3 and Table 4.

RESULTS
Extraction, PCR, sequencing

The results from the DNA extraction, PCR, and sequencing processes are summarised in Table 2. For some of the eight Cytb fragments, DNA amplification was not sufficient, which resulted in gaps in the sequence for a few specimens (Table 2). Upon checking the traces in CodonCodeAligner, we observed in our alignment that Y (C or T; n = 50) and R (G or A; n = 19) are the most common ambiguities. These nucleotide substitutions are most likely caused by hydrolytic deamination. This is a process by which the deamination of cytosine residues to form uracil residues, 5-methyl-cytosine residues to form thymine residues, or adenine residues to form hypoxanthine residues in the template DNA strand will be misread during the PCR process when a new DNA strand is synthesised. In turn, this leads
Figure 4  Consensus tree of the unpartitioned Bayesian Analyses (BI-1140-unpart). Values represent posterior probabilities (PP), and if applicable, bootstrap (BS) support from the ML analysis is shown. Only values larger than 70% (PP) and 50% (BS) are displayed. If the support was not above 70% or 50%, but the split was present in one of the analyses; this is indicated by an “–”.

(continued on next page...)
to evident C → T or G → A substitutions (Hofreiter et al., 2001a; Pääbo et al., 2004; Briggs et al., 2007; Briggs et al., 2010). Across our samples, Y ambiguities occurred up to ten times per specimen, and R ambiguities occurred up to three times per specimen. These numbers represent a very small proportion of approximately 1% of the overall sequence length of 747 base pairs. We tested the impact of the ambiguities on the reconstruction and found that the ambiguities did not tremendously influence the phylogenetic signal of the samples. However, these ambiguities represent an additional uncertainty in the analyses.

**Phylogenetic analyses**

The results from seven analyses are summarised in Table 4 and Fig. 3. Of the full 1140-base-pair-long Cytb data set 593 characters are constant, 68 variable characters are parsimony-uninformative, and 479 characters are parsimony-informative. The analyses of the 1140-base-pair-long Cytb represent our primary results and are shown in Fig. 4. In addition to the Bayesian Inference analyses and the Maximum Likelihood analysis of the total Cytb data set (including the new sequences), we tested the impact of reduced data sets (569 characters and 747 characters, Bayesian Inference) and different partitioning schemes on the phylogenetic signal (BI-1140-unpart, BI-1140-part, ML-1140, BI-569-unpart, BI-747-unpart, BI-747-part; Table 4, Fig. 3, Figs. S2–S7).

We next re-analysed the complete mitochondrial genome alignment from Hassanin et al. (2012) for the subset of cervids (14904 base pairs, Bayesian Inference; BI-mtG, Fig. S1), because the authors stated that some of the nodes are not robust, as proven by previous re-analyses (Bibi, 2014). The re-analysis presented here (BI-mtG, Fig. S1) resulted in the support of a fully resolved topology, which is congruent with the topology in Hassanin et al. (2012).

Data partitioning of the 1140-base-pair-long Cytb data set and reduced data sets did not lead to contradictory results compared to unpartitioned analyses or larger data sets. Resolution and node supports generally decreased with decreasing alignment length (Fig. 3). Cervid lineages above the genus level were almost always recovered with all matrix sizes and partitioning schemes (Table 4). None of the topologies supportably contradicted each other; however, all topologies differed from each other to some extent at the tribe, genus, and/or species level. Compared to the Cytb-only topologies, the mitochondrial genome topology showed generally higher posterior probabilities (Figs. 3 and 4).

The monophyly of superordinate clades, Cervidae, Cervini, Muntiacini, and Capreolini (including Hydropotes), was supported in all topologies (Figs. 3 and 4, Figs. S1–S7, Table 4). In all but one topology (BI-569-unpart; Fig. 3G, Fig. S7), the monophyly of
Cervinae, was consistently supported (Fig. 3, Table 4). Odocoileini was weakly supported in three topologies (ML-1140, BI-1140-unpart, BI-mtG; Figs. 3A, 3B, 3D and 4, Figs. S1, S2, S4 and Table 4). Capreolinae, however, was supported as a monophyly in only one topology (BI-mtG, Fig. 3A, Fig. S1), and in the other topologies, the taxon splits unresolved into Odocoileini, Rangiferini (Rangifer), Alceini (Alces), and Capreolini (Capreolus, Hydropotes) (Fig. 3). Alceini and Capreolini sometimes formed a clade (Figs. 3A, 3C, 3D, 3E and 3F) or were unresolved (Figs. 3B and 3G). Systematic relationships of capreoline taxa showed marginal differences in each of our topologies.

The results at the genus and species levels are shown in Fig. 4 and Figs. S1–S7. The newly sequenced Muntiacus atherodes nested within Muntiacini, mostly polytomous, with two Muntiacus-clades. One clade consisted of M. muntjak, M. feae, and M. crinifrons, and the other consisted of M. truongsonensis, M. putaoensis, M. rooseveltorum, M. reevesi, and M. vuquangensis. Two topologies (BI-1140-unpart, ML-1140) indicated a poorly supported sister taxon relationship between M. muntjak and M. atherodes (Fig. 4, Figs. S2 and S4).

We found strong support in Cervini to place all four Rusa marianna specimens in a Philippine Rusa-clade, with Rusa alfredi in all but one topology (BI-569-unpart; Fig. 4, Figs. S1–S7).

The newly sequenced Mazama chunyi is consistently placed as a sister taxon to M. gouazoubira, whereas the three M. bricenii specimens are primarily a sister taxon to M. rufina.

The four P. mephistophiles specimens always form a clade, which is either a sister taxon to or nested within Odocoileini. Interestingly, they are not placed in a sister position to the mitochondrial genome sequence labelled P. mephistophiles from Hassanin et al. (2012). In none of our topologies did P. mephistophiles and P. puda form a sister taxon relationship, which makes the monophyly of the genus questionable. M. nemorivaga, M. rufina, M. bricenii, P. puda, and particularly P. mephistophiles occasionally take up positions outside the above proposed clades, thus underpinning their yet unsolved systematics.

Regardless of the controversies debated here and elsewhere regarding Odocoileini molecular systematics, topologies (in the literature and here, Figs. 3 and 4, Figs. S1–S7) show two consistently occurring subclades carrying phylogenetic signal within Odocoileini (e.g., Gilbert, Ropiquet & Hassanin, 2006; Duarte, Gonzalez & Maldonado, 2008; Hassanin et al., 2012). One subclade consists of Hippocamelus, Blastocerus, Ozotoceros, M. gouazoubira, M. chunyi, M. nemorivaga, and Pudu puda. The other subclade consists of Odocoileus, M. americana, M. bororo, M. nana, M. temama, M. pandora, M. rufina, and M. bricenii. Based on these results we establish two new subtribes Blastocerina and Odocoileina according to the rules of the ICZN (http://www.iczn.org/code). These two subtribes form the tribe Odocoileini and have Rangiferini as sister taxon.

**Blastocerina subtribus nova**

Type genus: Blastocerus Wagner, 1844

Higher taxa: Odocoileini—Capreolinae—Cervidae

The subtribe Blastocerina consists of the following species: Blastocerus dichotomus, Hippocamelus antisensis, Hippocamelus bisulcus, Mazama chunyi, Mazama gouazoubira,
Mazama nemorivaga, Ozotoceros bezoarticus, and Pudu puda (Fig. 4). Blastocerina refers to the clade originating from the most recent common ancestor of Blastocerus dichotomus (Illiger, 1815) and Pudu puda Molina, 1782. Pudu mephistophiles potentially falls within that clade, but more data are needed for a definite placement of this taxon.

**Odocoileina subtribus nova**

Type genus: *Odocoileus* Rafinesque, 1832

Higher taxa: Odocoileini—Capreolinae—Cervidae

The subtribe Odocoileina consists of Mazama americana, Mazama bororo, Mazama bricenii, Mazama nana, Mazama pandora, Mazama rufina, Mazama temama, Odocoileus hemionus, and Odocoileus virginianus (Fig. 4). Odocoileina refers to the clade originating from the most recent common ancestor of Odocoileus virginianus (Von Zimmermann, 1778–1783) and Mazama bricenii Thomas, 1908.

**DISCUSSION**

**Phylogenetic analyses**

Our results represent the most complete compilation of molecular data in terms of taxon sample for cervids to date. The thorough sampling enabled us to place the de novo sequenced species in topologies representing overall cervid systematics. We were able to solve some relationships but also discovered previously unknown issues. The data set excludes Muntiacus gongshanensis, for which only a very short tRNA sequence is available, and Axis calamianensis, M. montanus, M. puhoatensis, and M. vaginalis, for which no molecular data are available.

Our experiments with different matrix sizes, partitioning schemes, and models revealed that the resulting topologies do not dramatically differ from each other. However, we could observe that the resolution decreased with decreasing sequence length. All seven analyses recovered major clades within Cervidae (Table 4 and Fig. 3). These experiments were undertaken to single out strong phylogenetic signal and the significance thereof, which is consistent regardless of the data set sizes and parameter changes. We observed that taxa, which are generally unstable across topologies from different studies (e.g., Pitra et al., 2004; Gilbert, Ropiquet & Hassanin, 2006; Agnarsson & May-Collado, 2008; Hassanin et al., 2012), were the first to lose a supported systematic position with decreasing sequence length (Fig. 3 and Table 4). The partitioning scheme and model choice did not make as much difference as did the matrix size. As expected, partitioning did not necessarily lead to better resolved topologies or significantly better supported clades. However, some differences were observed comparing maximum likelihood with Bayesian inference methods (Figs. 3 and 4).

The topology resulting from re-analysis of the mitochondrial genome sequences (BI-mtG) representing the largest sequence length is fully resolved and has the highest overall support values. The shortest data set (BI-569-unpart), although less well resolved, recovered all higher-level lineages and is in most points congruent with the other topologies based on larger data sets (Table 4 and Fig. 3). These different analyses enabled us to examine the significance of the individual resulting topologies.
**Muntiacus atherodes**

The species diversity of Muntiacini is the least covered among cervid subclades in molecular phylogenetic analyses. Muntiacini comprises muntjacs (*Muntiacus*) and the tufted deer (*Elaphodus*), includes the smallest members of Cervinae (40 to 70 cm shoulder height), and inhabits Southeast Asia and Eastern China (Mattioli, 2011). The systematic relationships within Muntiacini in our topologies (Fig. 4) are largely congruent with most recent studies and are the least controversial in molecular cervid systematics (e.g., Pitra et al., 2004; Gilbert, Ropiquet & Hassanin, 2006; Agnarsson & May-Collado, 2008; Hassanin et al., 2012). Here, *M. crinifrons* and *M. feae* are always sister taxa, and when the resolution is sufficiently high, *M. muntjak* is a sister taxon to both of them. In our topologies, *M. putaoensis*, *M. rooseveltorum*, *M. truongsonensis*, and *M. vuquangensis* always form a clade. Most often, with *M. reevesi* is a sister taxon to that clade, but occasionally, *M. reevesi* is sister taxon to all other muntjacs (BI-747-part, BI-569-unpart). Due to the consistent position of *M. muntjak* 2 (AF042718) as sister taxon to *M. truongsonensis*, we suggest re-confirming this sequence.

The monotypic *Elaphodus cephalophus*, which is distributed in southeast China, is always a sister taxon to all muntjacs in both our topologies and previously published trees (Pitra et al., 2004; Gilbert, Ropiquet & Hassanin, 2006; Agnarsson & May-Collado, 2008; Hassanin et al., 2012).

Because of the presumed primitive antler morphology of *M. atherodes* (Groves & Grubb, 1982), its systematic position was hypothesised to be between *Elaphodus cephalophus* and the *Muntiacus*-clade, which is not supported by our results. The newly sequenced holotype specimen of *M. atherodes* is nested within muntjacs, unresolved in a polytomy in most of our topologies. However, some results indicate a potential closer relationship to *M. muntjak* than to any other muntjac. The predominant separate placement from all other *Muntiacus* spp. is an interesting outcome that strengthens the species status of *M. atherodes*.

Several authors assumed the sympatric existence of a second muntjac species on Borneo that was separate from *M. muntjak* (Kohlbrugge, 1895; Lyon Jr, 1911; Van Bemmel, 1952; Hill, 1960) before Groves & Grubb (1982) eventually established *M. atherodes* based on a skin and the holotype skull sampled for the present study. The endemic *M. atherodes* differs from *M. muntjak* in colouration and has smaller, simpler antlers, and the latter has a much wider distribution across Southeast Asia and Southern China (Groves & Grubb, 1982).

Though unsupported, the potential close systematic relationship of *M. atherodes* and *M. muntjak* would be logical based on the endemic occurrence of *M. atherodes* on Borneo. *M. atherodes* and *M. muntjak* could have diverged from a common ancestor on Borneo via sympatric speciation and with a later invasion of *M. muntjak* to the mainland.

Alternatively, *M. muntjak* could have invaded Borneo during the sea level fluctuations in the Plio-Pleistocene (Voris, 2000; Meijaard, 2003; Woodruff, 2003; Meijaard & Groves, 2004; Bibi & Métais, 2016), resulting in the allopatric speciation of *M. atherodes* and its isolation from the mainland populations during the end-Pleistocene sea level rise.

The high sea levels in the early Pliocene split the Thai-Malayan Peninsula into two landmasses, which separated Indochinese from Sundaic faunas (Woodruff, 2003). This most likely had a large influence on the evolution of Southeast Asian cervids and probably
occurred again later during the Pliocene (Meijaard & Groves, 2004). Sea level changes in the Malay Archipelago were important for faunal dispersals. Low sea levels allowed species to spread to landmasses, which would become islands with rising sea levels, resulting in isolation of populations.

Detailed descriptions and maps for sea level changes of Southeast Asia can be viewed in Voris (2000) and Meijaard (2003).

**Rusa marianna**

In the literature, there is a broad consensus about the systematic relationships within Cervini. However, the taxonomy of Cervus s. l. is indeed complicated (Randi et al., 2001). The controversy primarily concerns delimitations of genera and/or subgenera. Rusa, Rucervus, Przewalskium (= Cervus) albirostris, and Cervus are occasionally treated as subgenera of the genus Cervus, whereas Axis, Elaphurus, and Dama are normally treated as separate genera (Groves & Grubb, 1987; Randi et al., 2001). Here, we refer to Rucervus and Rusa as individual genera and refer to Przewalskium albirostris as Cervus albirostris.

The four species of Rusa, R. alfredi, R. marianna, R. timorensis, R. unicolor, inhabit India, Indochina and the Malay-Archipelago (Grubb & Groves, 1983; Mattioli, 2011). R. unicolor is the largest oriental deer and has a highly fragmentary distribution from southern Nepal, India and Sri Lanka along the southern Himalayas through to mainland Southeast Asia and many of the Greater Sunda islands (Timmins et al., 2008; Leslie, 2011). R. timorensis is endemic to the Indonesian islands Bali and Java (Hedges et al., 2008). Rusa alfredi is one of the rarest deer species according to the IUCN Red List (IUCN, 2015) and is endemic to Panay and Negros (Western Visayan Islands, Central Philippines) (Oliver et al., 2008).

In contrast, Rusa marianna is more widely distributed across most of the Philippine Islands, with the exceptions of the Negros-Panay, Sulu and Palawan Faunal Region, the Babuyan/Batanes groups, and other isolated islets (MacKinnon, Ong & Gonzales, 2008).

The four newly sequenced individuals of Rusa marianna are positioned to be closely related to each other in a distinct clade. Two of the individuals are in a polytomy with the other Philippine species, Rusa alfredi, and two form a clade, which is a sister taxon to the polytomy (Fig. 4). Our topology supports the hypothesis that the two Philippine Rusa species are closely related and are sister taxon to R. timorensis and R. unicolor.

Investigations by Grubb & Groves (1983) showed that interpreted relationships within Rusa are controversial. Rusa timorensis and R. unicolor are sister taxa supported in all our topologies (Fig. 4, Figs. S1–S7), and this clade is in a polytomy with the Cervus-clade (including C. albirostris) and the R. alfredi-R. marianna-clade. A close relationship between Rusa and C. albirostris was already suggested by Flerov (1952) based on morphological evidence and a supposed divergence of C. albirostris from Rusa in the Late Pliocene.

The evident phenotypic separation of spotted (R. alfredi) and non-spotted (R. marianna) Rusa deer on the Philippines suggests two invasion events (Grubb & Groves, 1983), but the missing molecular data for R. marianna have prohibited further explanations. Grubb & Groves (1983) suggested a Southeast Asian mainland common ancestor from which a peripheral population diverged by evolving into R. timorensis. Later, a population of those colonised the Philippines twice at early and later stages in diversification, evolving
into *R. alfredi* and *R. marianna*. *R. unicolor* evolved there but failed a third colonisation on additional Philippine Islands and dispersed northwards to the mainland. *Meijaard & Groves (2004)* pointed to the likely high impact of Plio-Peistocene sea level fluctuations on Southeast Asian cervid dispersal and speciation.

However, the suggested speciation of *R. marianna* and *R. alfredi* is not clearly evident from our topologies, where *R. alfredi* appears to be a subgroup of *R. marianna* rather than a sister taxon. More data are needed to unambiguously solve their relationships.

### Odocoileini

Odocoileini represents the most controversial subclade of extant cervids. They consistently split into two subclades in both our current results and previously published phylogenetic trees. For these two subclades we established the new subtribes Blastocerina and Odocoileina (see above). However, within each of these subclades, systematic relationships are not yet solved. The recent divergence of modern neotropical Odocoileini from extinct Eurasian Capreolinae and related insufficient genomic diversity available to solve systematic relationships could be the reason (*Vislobokova, 1980*). All genera except for *Odocoileus* are endemic to South America, and their ancestors reached the continent via the Panamanian Isthmus in the Pliocene (5–2.5 million years ago) (*Webb, 2000*; *Gilbert, Ropiquet & Hassanin, 2006*). The first fossil appearances are known from no longer than approximately 2.4 million years ago (*Webb, 2000*). The consistent split of Blastocerina and Odocoileina potentially represents an asynchronous dispersal history via two invasion events.

Furthermore, our study revealed dubious relationships between available *Hippocamelus* sequences. All of our topologies (Fig. 4, Figs. S2–S7) show that two *H. antisensis* sequences (*H. antisensis* 1; JN632646, NC_020711 (*Hassanin et al., 2012*)) are a sister taxon to *H. bisulcus*. However, the other two sequences (*H. antisensis* 2; DQ379307 (*Gilbert, Ropiquet & Hassanin, 2006*) and GU190862 (*Fuentes-Hurtado et al., 2011*)), are a sister taxon to *Ozotoceros* in all of our topologies (Fig. 4, Figs. S2–S7). This is a critical issue, although its resolution is beyond the scope of this study; however, we found it important to point to this drawback in the base data and suggest re-confirmation of all four sequences.

Systematics of the two dwarfed genera, *Mazama* and *Pudu*, whose small body size and simplified antlers are interpreted as secondary adaptations to dense vegetation (*Geist, 1998; Mattioli, 2011*), are particularly uncertain. Their habitat use and their decline in individual numbers makes it increasingly difficult to obtain enough data to resolve systematic issues from some of the species (see below).

### Pudu

Pudus are the smallest living deer (25 to 40 cm shoulder height) and the smallest New World hoofed mammals (*Hershkovitz, 1982; Mattioli, 2011*). It is difficult to distinguish both *pudu* species from sympatric small deer species (*Mazama*) based only on the phenotype, without direct comparison (*Hershkovitz, 1982; Jiménez, 2011*). *Pudu* and *Mazama* likely represent divergent lineages of small odocoilein deer (*Hershkovitz, 1982*). Although the origin of pudus is unknown, *Hershkovitz (1982)* stated that *P. mephistophiles* has more primitive phenotypical features than *P. puda*. 
Pudu was assumed to be polyphyletic (Hassanin et al., 2012). Whereas P. puda has been well-sampled and studied, information for P. mephistophiles is scarce. In all of our topologies (Fig. 4, Figs. S1–S7), the four newly sequenced specimens of Pudu mephistophiles, including the holotype, form a well-supported clade. However, the position of that clade is variable. In four topologies (BI-1140-unpart, BI-747-part, BI-747-unpart, BI-569-unpart; Fig. 4, Figs. S2, S5, S6 and S7), the clade is a sister taxon to all other Odocoileini and Rangiferini; in one topology (ML-1140; Fig. S4), it is a sister taxon to all Blastocerina with poor support; and in one topology (BI-1140-part; Fig. 3C, Fig. S3), it is placed in an unresolved position with other Odocoileini clades and Rangiferini. The placement of the individual Pudu mephistophiles specimen published prior to our study in Hassanin et al. (2012) (JN632691) is not close to the P. mephistophiles-clade in our topologies. Instead, it is placed as a sister taxon to Mazama rufina (Fig. 4, Figs. S1–S7) and confirms Hassanin et al.’s (2012) suspicion that it might in fact be a misidentified Mazama rufina and is neglected for further interpretation. The holotype specimen included in the four new P. mephistophiles samples substantiates that suspicion. In all but one topology (BI-569-unpart), P. puda is a sister taxon to all other Blastocerina, which is congruent with Hassanin et al. (2012) and Agnarsson & May-Collado (2008). In Duarte, González & Maldonado (2008), however, its position was unresolved. The placement of P. mephistophiles separate from its congeneric P. puda in most topologies suggests polyphyly of the genus.

**Mazama**
The genus Mazama comprises several species of small- to medium-sized deer (40 to 80 cm shoulder height) (Hershkovitz, 1959; Hershkovitz, 1982; Mattioli, 2011). The current distribution of Mazama ranges from Southern Mexico to Argentina (IUCN Red List, Mattioli, 2011; González et al., 2009).

Since the first description of Mazama pita Rafinesque, 1817 (= Moschus americanus Erxleben, 1777), the genus has been subject to taxonomic controversies. Allen (1915) recognised 18 species of Mazama; Cabrera (1960) reduced these to four species, i.e., M. chunyi, M. gouazoubira, M. nana, and M. rufina. Czernay (1987) established two more species, M. americana and M. bricenii, whereas Groves & Grubb (1987) considered M. temama a possible separate species based on cytogenetic differences. Medellín, Gardner & Marcelo Aranda (1998) revised M. pandora as a separate species based on differences in the skulls and skins. Rossi (2000) established M. nemorivaga as a fourth sympatric species in Brazil (together with M. americana, M. nana, M. gouazoubira). Duarte (1992) described M. bororo based on karyotype differences, which adds up to ten Mazama species being widely accepted today (IUCN Red List, Mattioli, 2011; González et al., 2009). More recently, Abril & Duarte (2008) recognised only eight species (M. americana, M. bororo, M. chunyi, M. gouazoubira, M. nana, M. nemorivaga, M. pandora, and M. rufina), whereas Groves & Grubb (2011) listed 24 different species of Mazama. Most of the species share phenotypic similarities, which makes their discrimination almost impossible; however, there are differences in overall body size, coat colour, and/or karyotype (González et al., 2009).
Recently, polyphyly of *Mazama* was observed (Duarte, González & Maldonado, 2008; Hassanin et al., 2012). Within Odocoileina, Duarte, González & Maldonado (2008) found a separation of the genus into a mixed *Mazama americana*-clade that included *M. bororo* and *M. nana*. *M. americana* appeared polyphyletic because there was an additional clade consisting exclusively of *M. americana* as a sister taxon to *Odocoileus* and the mixed *M. americana*-clade (Duarte, González & Maldonado, 2008). Hassanin et al. (2012) found *M. americana* to be monophyletic and a sister taxon to *Odocoileus*. *M. rufina* is a sister taxon to the *Mazama-Odocoileus*-clade (Hassanin et al., 2012).

Within Blastocerina there were two clades: a *Mazama gouazoubira*-clade and a *M. nemorivaga*-clade. Their position varies from study to study (Agnarsson & May-Collado, 2008; Duarte, González & Maldonado, 2008; Hassanin et al., 2012).

In our topologies, within Odocoileina, the mixed *Mazama americana*-clade that includes the sequences indicated as *M. americana* 1–3 is supported (Fig. 4) and has the most stable position, forming the sister taxon to the *Odocoileus*-clade. The pure *M. americana*-clade found by Duarte, González & Maldonado (2008) is represented in our topology by the sequences indicated as *M. americana* 4 and *M. americana* 5.

*M. rufina* is nested within Odocoileina and is a sister taxon to the *Mazama-Odocoileus*-clade (BI-1140-unpart, BI-1140-part, ML-1140; Figs. 3 and 4, Figs. S2, S3 and S4) or is placed in resolved or unresolved positions outside Odocoileina but within Odocoileini (BI-747-unpart, BI-747-part, BI-569-unpart; Fig. 3, Figs. S5, S6 and S7).

*M. gouazoubira* is either a sister taxon to both *Hippocamelus* species (BI-747-unpart, BI-569-unpart; Fig. 4, Figs. S5 and S7), or *Blastocerus* is placed between *Hippocamelus* and *M. gouazoubira*. *M. gouazoubira* itself is polyphyletic in our topologies (Fig. 4), and a reconfirmation of the *M. gouazoubira* 2 sequence (DQ379308 (Gilbert, Ropiquet & Hassanin, 2006)) is suggested.

Finally, the *M. nemorivaga*-clade is mostly nested within Blastocerina or is placed unresolved within Odocoileini (BI-747-part, BI-569-unpart).

In our study, *M. temama* and *M. pandora* were included in a species-rich phylogenetic analysis of cervids with palaearctic and neotropical species for the first time. Similarly to recent results of Escobedo-Morales et al., 2016, our results show that *M. temama* is always within Odocoileina as a sister taxon to the mixed *M. americana*-clade. In Escobedo-Morales et al., 2016 and in our topologies, *M. pandora* is consistently placed within Odocoileina as a sister taxon to *Odocoileus*.

This also indicates a critical issue concerning the dispersal history of South American cervids. The placement of the *M. americana*-splits in Fig. 4 can be alternatively interpreted as a paraphyletic *M. americana*-clade, within which all other species are nested, i.e., *Odocoileus* sp., *M. pandora*, *M. temama*, *M. nana*, and *M. bororo*. However, the placement of *M. temama* disrupts the continuous genealogy of *M. americana*. Together with the clade consisting of *M. rufina* and *M. bricenii* (see below), Odocoileina is basically a *Mazama*-clade, within which *Odocoileus* diverged and *Mazama* diversified into several species. This scenario would strongly question the long-held assumption that *Odocoileus* was the first cervid to immigrate to South America and diversify into the extant South American species (Anderson & Wallmo, 1984; Smith, 1991; Geist, 1998) (see also Escobedo-Morales et al., 2016).
Our results from sequencing *M. chunyi* show a sister taxon relationship with *M. gouazoubira* within Blastocerina in all our topologies (Fig. 4). The newly sequenced *Mazama bricenii* specimens are always placed in a sister taxon position to *M. rufina* in our topologies but exist as a monophyletic group in only one topology (BI-569-unpart; Fig. 3, Fig. S7).

In two topologies, the specimen BMNH 1908.6.24.5 is placed isolated from the other two specimens (BMNH 1913.4.24.3, BMNH 1934.9.10.228), which remain sister taxa to *M. rufina*. Specifically, in one topology, BMNH 1908.6.24.5 is in an unresolved position within Odocoileina (BI-747-part; Fig. 3, Fig. S6) and is positioned as a sister taxon to *M. chunyi* in the other topology (BI-1140-part; Fig. 3, Fig. S3).

Mattioli (2011) listed *M. bricenii* and *M. chunyi* as subspecies of *M. rufina*. The *Mazama bricenii* specimen BMNH 1934.9.10.228 was originally assigned to *M. rufina*. Additionally, its sampling locality in Ecuador is outside the assumed current distribution of *M. bricenii* (Fig. 2 and Table 2) and thus makes the revised affiliation to *M. bricenii* questionable. *M. bricenii* is scarcely distributed in Northeast Colombia and West Venezuela, whereas *M. rufina* is distributed along the Andes from central Colombia to Ecuador and North Peru (Weber & González, 2003; Lizcano, Álvarez & Delgado-V, 2010). This distribution is intermediate between the distribution of *M. bricenii* and *M. chunyi*. The latter is certainly known from South Peru and North Bolivia based on isolated museum specimen localities and rare sightings in the wild. Equally scarce is information on the biology and ecology of these species (Rumiz & Pardo, 2010). The results of the most recent study on systematic relationships of *M. bricenii* based on *Cytb* confirm our results and suggest that *M. bricenii* is a junior synonym of *M. rufina* (Gutiérrez et al., 2015).

Despite the extensive taxonomic and phylogenetic interest in the genus *Mazama* due to unsolved questions, the taxon remains enigmatic (e.g., Duarte & Merino, 1997; Medellín, Gardner & Marelo Aranda, 1998; Duarte & Jorge, 2003; Weber & González, 2003; Duarte, González & Maldonado, 2008; González et al., 2009). In particular, the high intraspecific variability in *M. americana* and *M. gouazoubira* stimulated additional taxonomic and genetic research on the genus (see Weber & González, 2003). The systematics of *M. americana* is particularly problematic because even the species appears polyphyletic with possible cryptic species (Duarte, González & Maldonado, 2008; Abril et al., 2010).

Abril et al. (2010) showed that *M. americana* exhibits an extensive karyotype variation and found two distinct clades within *M. americana* sampled across Brazil. They also found that one clade is more closely related to *M. bororo* and *M. nana*, presumably corresponding to *M. americana* 1–3 in our topology, than to the second (pure) clade of *M. americana* (Fig. 4). Additionally, the genetic distance between the *M. americana*-clades was higher than that between *M. nana* and *M. bororo*. This suggests two separation events in the two lineages of *M. americana* (Abril et al., 2010). There is the potential that even more species are hidden in both the *M. americana*-complex and the *M. gouazoubira*-complex (Weber & González, 2003). Cytogenetics seems to be the most reliable technique for distinguishing between sympatric species (Vogliotti & Duarte, 2009). Much more data and thorough research on *Mazama* are needed to shed additional light on their complex systematic relationships.
CONCLUSION

The taxonomically most extensive molecular phylogenetic data set for cervids compiled to date enabled us to undertake phylogenetic analyses to answer and test the initial questions and hypotheses: (1) *Mazama bricenii* is closely related to *M. rufina* and is more closely related to the *M. americana*-clade than to the *M. gouazoubira*-clade. However, from our topology, we infer that *M. rufina* is a subclade of *M. bricenii*. It cannot be excluded that these two taxa may represent the same species with *M. rufina* as the senior synonym. *Mazama chunyi* forms a sister taxon relationship with *M. gouazoubira* and can thus be assigned to the *M. gouazoubira*-clade. The discovery of a fifth clade (*M. pandora*) shows that the polyphyly and systematic relationships within *Mazama* are even more complex than previously thought and remain a challenge to address in future research. (2) *Muntiacus atherodes* is supported to be a valid species distinct from other *Muntiacus* spp. However, its systematic position cannot be resolved with certainty, but the maximum likelihood analysis indicates that it might be more closely related to the sympatric *M. muntjak* than to any other muntjac. (3) The Philippine rusine deer *R. marianna* and *R. alfredi* form a monophyletic clade and are sister taxon to a clade containing the other rusine deer, *R. timorensis* and *R. unicolor* and to the *Cervus*-clade. Our results indicate that *R. alfredi* forms a subclade of *R. marianna* rather than its sister taxon. (4) The genus *Pudu* appears to be polyphyletic, with *P. puda* nested within the Blastocerina and *P. mephistophiles*, thereby forming a monophyletic group in a yet-unresolved position.

Based on our topologies and previous work, we established here the new subtribes Blastocerina and Odocoileina, which form Odocoileini. A revision of the current taxonomy based on comparison of phenotypic and genotypic traits is desirable for future research on cervid systematics.

ACKNOWLEDGEMENTS

We thank Richard Sabin at the Natural History Museum London (BMNH) for providing access to the collections and permission to sample from specimens, including type material, and Tracy Heath (BMNH) for mailing the samples to Munich. We are grateful to Frieder Mayer for permission to sample all requested specimens at the Museum für Naturkunde in Berlin (ZMB) and Nora Lange for sampling the specimens and mailing the samples. We thank Gabriele Büttner for assistance with laboratory work. We also thank Frank Zachos, Robert Asher and two anonymous reviewers for their constructive comments on the manuscript.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was funded by the German Research Foundation (DFG) RO 1197/7-1. Additionally, NS Heckeberg was funded by the German Academic Exchange Service (DAAD) D/11/42358 and the Elitenetzwerk Bayern. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Grant Disclosures
The following grant information was disclosed by the authors:
German Research Foundation: RO 1197/7-1.
German Academic Exchange Service: D/11/42358.
Elitenetzwerk Bayern.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
- Nicola S. Heckeberg conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Dirk Erpenbeck, Gert Wörheide and Gertrud E. Rössner conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

DNA Deposition
The following information was supplied regarding the deposition of DNA sequences:
European Nucleotide Archive: LT546647–LT546659.

Data Availability
The following information was supplied regarding data availability:
All alignments and analyses information are deposited at Open Data LMU
doi: 10.5282/ubm/data.96.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.2307#supplemental-information.

REFERENCES
Abril VV, Carnelossi EAG, González S, Duarte JMB. 2010. Elucidating the Evolution of the red brocket deer Mazama americana complex (Artiodactyla; Cervidae). Cytogenetic Genome Research 128:177–187 DOI 10.1159/000298819.
Abril VV, Duarte JMB. 2008. Chromosome polymorphism in the Brazilian dwarf brocket deer, Mazama nana (Mammalia, Cervidae). Genetics and Molecular Biology 31(1):53–57.
Agnarsson I, May-Collado LJ. 2008. The phylogeny of Cetartiodactyla: the importance of dense taxon sampling, missing data, and the remarkable promise of cytochrome b to provide reliable species-level phylogenies. Molecular Phylogenetics and Evolution 48:964–985 DOI 10.1016/j.ympev.2008.05.046.
Allen GM. 1915. Notes on American deer of the genus Mazama. Bulletin American Museum of Natural History 34:521–553.
Anderson AE, Wallmo OC. 1984. Odocoileus hemionus. Mammalian Species 219:1–9.
Bibi F. 2014. Assembling the ruminant tree: combining morphology, molecules, extant taxa, and fossils. *Zitteliana B* 32:197–211.

Bibi F, Métais G. 2016. Evolutionary history of the large herbivorous of south and southeast Asia (Indomalayan Realm). In: Areshtani FS, Sankaran M, eds. *The ecology of large herbivores in south and southeast Asia*. Dordrecht, Heidelberg, Berlin: Springer, 15–88.

Briggs AW, Stenzel U, Johnson P, Green RE, Kelso J, Prüfer K, Meyer M, Krause J, Ronan MT, Lachmann M, Pääbo S. 2007. Patterns of damage in genomic DNA sequences from a Neandertal. *Proceedings of the National Academy of Sciences of the United States of America* 104(37):14616–14621 DOI 10.1073/pnas.0704665104.

Briggs AW, Stenzel U, Meyer M, Krause J, Kircher M, Pääbo S. 2010. Removal of deaminated cytosines and detection of in vivo methylation in ancient DNA. *Nucleic Acids Research* 38(6):e87 DOI 10.1093/nar/gkp1163.

Brooke V. 1878. On the classification of the Cervidae, with a synopsis of the existing species. *Proceedings of the Zoological Society of London* 1878:883–928.

Cabrera A. 1960. Catálogo de los mamíferos de América del Sur. *Revista Museo Argentino Bernardino Rivadavia* 4:309–732.

Croitor R. 2014. Deer from Late Miocene to Pleistocene from Western Palearctic: matching fossil record and molecular phylogeny data. *Zitteliana B* 32:115-153.

Czernay S. 1987. *Die Spiesshirsche und Pudus. Die neue Brehm-Bücherei* 581. Wittenberg Lutherstadt: Ziemsen Verlag.

Duarte JMB. 1992. Aspectos taxonômicos e citogenéticos de algumas espécies de cervídeos brasileiros. MSc Thesis, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, Brazil.

Duarte JMB, González S, Maldonado JE. 2008. The surprising evolutionary history of South American deer. *Molecular Phylogenetics and Evolution* 49(1):17–22 DOI 10.1016/j.ympev.2008.07.009.

Duarte JMB, Jorge W. 2003. Morphologic and Cytogenetic Description of the small red brocket (*Mazama bororo* Duarte,1996) in Brazil. *Mammalia* 67(3):403–410 DOI 10.1515/mamm.2003.67.3.403.

Duarte JMB, Merino ML. 1997. Taxonomia e evolução. In: Duarte JMB, ed. *Biologia e conservação de cervídeos Sul-Americanos: Blastocerus, Ozotoceros e Mazama*. Jaboticabal: Fundação de Estudos e Pesquisas em Agronomia, Medicina Veterinária e Zootecnia, 2–21.

Duckworth JW, Robichaud WG, Timmins RJ. 2008. *Rucervus schomburgki*. The IUCN red list of threatened species. Version 2014.3. Available at [www.iucnredlist.org](http://www.iucnredlist.org) (accessed on 17 April 2015).

Erxleben JCP. 1777. *Systema regni animalis per classes, ordines, genera, species, varietates, cum synonymia et historia animalium. Classis I. Mammalia*. Weygandianis, Lipsiae, 636 pp.

Escobedo-Morales LA, Mandujano S, Eguiarte LE, Rodriguez-Rodriguez MA, Maldonado JE. 2016. First phylogenetic analysis of Mesoamerican brocket deer *Mazama pandora* and *Mazama temama* (Cetartiodactyla: Cervidae) based on mitochondrial
sequences: implications on neotropical deer evolution. *Mammalian Biology* 81(3):303–313.

Flerov KK. 1952. *Fauna of the USSR., Mammals 1(2). Musk deer and deer*. Moscow: Academy of Sciences of USSR.

Fuentes-Hurtado M, Marin JC, Gonzalez-Acuna D, Verduco C, Vidal F, Vianna JA. 2011. Molecular divergence between insular and continental *Pudu* deer (*Pudu puda*) populations in the Chilean Patagonia. *Studies in Neotropical Fauna and the Environment* 46(1):23–33 DOI 10.1080/01650521.2010.537906.

Geist V. 1998. *Deer of the world: their evolution, behaviour, and ecology*. Mechanicsburg: Stackpole Books, 421 pp.

Gilbert C, Ropiquet A, Hassanin A. 2006. Mitochondrial and nuclear phylogenies of Cervidae (Mammalia, Ruminantia): systematics, morphology, and biogeography. *Molecular Phylogenetics and Evolution* 40(1):101–117 DOI 10.1016/j.ympev.2006.02.017.

González S, Maldonado JE, Ortega J, Talarico AC, Bidegaray-Batista L, Garcia JE, Duarte J. 2009. Identification of the endangered small red brocket deer (*Mazama bororo*) using noninvasive genetic techniques (Mammalia; Cervidae). *Molecular Ecology Resources* 9:754–758 DOI 10.1111/j.1755-0998.2008.02390.x.

Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution* 27(2):221–224 DOI 10.1093/molbev/msp259.

Groves CP, Grubb P. 1982. The species of muntjac (genus *Muntiacus*) in Borneo: unrecognised sympatry in tropical deer. *Zoologische Mededelingen* 56:203–216.

Groves CP, Grubb P. 1987. Relationships of living deer. In: Wemmer CM, ed. *Biology and management of the Cervidae*. Washington D.C.: Smithsonian Institution Press, 21–59.

Groves CP, Grubb P. 2011. *Ungulate taxonomy*. Baltimore: Johns Hopkins University Press, 309 pp.

Grubb P, Groves CP. 1983. Notes on the taxonomy of the deer (Mammalia, Cervidae) of the Philippines. *Zoologischer Anzeiger* 210:119–144.

Gutiérrez EE, Maldonado JE, Radosavljevic A, Molinari J, Patterson BD, Martínez-C JM, Rutter AR, Hawkins MTR, García FJ, Helgen KM. 2015. The taxonomic status of *Mazama bricenii* and the significance of the táchira depression for mammalian endemism in the Cordillera de Mérida, Venezuela. *PLoS ONE* 10(6):e0129113 DOI 10.1371/journal.pone.0129113.

Hassanin A, Delsuc F, Ropiquet A, Hammer C, Jansen van Vuureen B, Matthee C, Ruiz-Garcia M, Catzeflis F, Areskoug V, Thanh Nguyen T, Couloux A. 2012. Pattern and timing of diversification of Cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes. *Comptes Rendus Biologies* 335(1):32–50 DOI 10.1016/j.crvi.2011.11.002.

Hassanin A, Douzery EJP. 2003. Molecular and morphological phylogenies of Ruminantia and the alternative position of the moschidae. *Systematic Biology* 52:206–228 DOI 10.1080/10635150390192726.
Hedges S, Duckworth JW, Timmins RJ, Semiaidi G, Priyono A. 2008. *Rusa timorensis*. The IUCN red list of threatened species. Version 2014.3. Available at www.iucnredlist.org (accessed on 17 April 2015).

Hernández-Fernández M, Vrba ES. 2005. A complete estimate of the phylogenetic relationships in Ruminantia: a dated species-level supertree of the extant ruminants. *Biological Reviews* 80:269–302 DOI 10.1017/S14647931040006670.

Hershkovitz P. 1959. A new species of South American brocket, genus *Mazama* (Cervidae). *Proceedings of the Biological Society Washington* 72:45–54.

Hershkovitz P. 1982. Neotropical Deer (Cervidae) Part I. Pudus, Genus *Pudu* Gray. *Fieldiana. Zoology, New Series* 11:1–86.

Hill JE. 1960. The Robinson Collection of Malaysian mammals. *Bulletin of the Raffles Museum* 29:1–112.

Hofreiter M, Jaenicke V, Serre D, Von Haeseler A, Pääbo S. 2001a. DNA sequences from multiple amplifications reveal artifacts induced by cytosine deamination in ancient DNA. *Nucleic Acids Research* 29(23):4793–4799 DOI 10.1093/nar/29.23.4793.

Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S. 2001b. Ancient DNA. *Nature Reviews, Genetics* 2:353–359.

Hughes S, Hayden TJ, Douady CJ, Tougaard C, Germonpré M, Stuart A, Lbova L, Carden RF, Hänni C, Say L. 2006. Molecular phylogeny of the extinct giant deer, *Megaloceros giganteus*. *Molecular Phylogenetics and Evolution* 40:285–291.

Illiger J. 1815. *Überblick der Säugethiere nach ihrer Vertheilung über die Welttheile: gelesen in der Akademie der Wissenschaften in Berlin am 28. Febr. 1811*. Realschul-Buchhandlung.

IUCN. 2015. The IUCN red list of threatened species. Version 2015.4. Available at http://www.iucnredlist.org (accessed on 20 December 2015).

Jiménez JE. 2011. Chapter 14 Southern *pudu* *Pudu puda* (Molina, 1782). In: Duarte JMB, González S, eds. Neotropical cervidology: biology and medicine of Latin American deer. Jaboticabal and Gland: Funep in collaboration with IUCN, 140–150.

Kohlbrugge JHF. 1895. *Zoogdieren van Zuid-Oost Borneo*. *Natuurkundig tijdschrift voor Nederlandsch Indië* 55:176–200.

Kuznetsova M, Kholodova M, Danilkin A. 2005. Molecular phylogeny of deer (Cervidae: Artiodactyla). *Russian Journal of Genetics* 41:742–749 DOI 10.1007/s11177-005-0154-1.

Lanfear R, Calcott B, Ho SYM, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29(6):1695–1701 DOI 10.1093/molbev/mss020.

Leslie DM. 2011. *Rusa unicolor* (Artiodactyla: Cervidae). *Mammalian Species* 43(871):1–30 DOI 10.1644/871.1.

Lister AM, Edwards CJ, Nock DAW, Bunce M, Van Pijlen IA, Bradley DG, Thomas MG, Barnes I. 2005. The phylogenetic position of the ‘giant deer’ *Megaloceros giganteus*. *Nature* 438:850–853 DOI 10.1038/nature04134.

Lizcano DJ, Álvarez SJ, Delgado-V CA. 2010. Chapter 19 Dwarf red brocket *Mazama rufina* (Pucheran 1951). In: Duarte JMB, González S, eds. Neotropical cervidology:
biology and medicine of Latin American deer. Jaboticabal and Gland: Funep in collaboration with IUCN, 181–184.

Lyon Jr MW. 1911. Mammals collected by Dr. Abbott in Borneo and some of the small adjacent islands. Proceedings of the United States National Museum 40:53–146 DOI 10.5479/si.00963801.40-1809.53.

MacKinnon J, Ong P, Gonzales JC. 2008. Rusa marianna. The IUCN red list of threatened species. Version 2014.3. Available at www.iucnredlist.org (accessed on 17 April 2015).

Maddison WP, Maddison DR. 2011. Mesquite: a modular system for evolutionary analysis. Version 2.75. Available at http://mesquiteproject.org.

Marcot JD. 2007. Molecular phylogeny of terrestrial artiodactyls. Conflicts and resolution. In: Prothero DR, Foss SE, eds. The evolution of artiodactyls. Baltimore: Johns Hopkins University Press, 4–18.

Mattioli S. 2011. Family Cervidae (Deer). In: Wilson DE, Mittermeier RA, eds. Handbook of the mammals of the world Volume 2. Barcelona: Lynx Edicion, 886 pp.

Medellín RA, Gardner AL, Marelo Aranda J. 1998. The taxonomic status of the Yucatan brown Brocket Mazama pandora (Mammalia: Cervidae). Proceedings of the Biological Society of Washington 111:1–14.

Meijaard E. 2003. Mammals of south-east Asian islands and their Late Pleistocene environments. Journal of Biogeography 30:1245–1257 DOI 10.1046/j.1365-2699.2003.00890.x.

Meijaard E, Groves CP. 2004. Morphometrical relationships between South-east Asian deer (Cervidae, tribe Cervini): evolutionary and biogeographic implications. Journal of Zoology 263:179–196 DOI 10.1017/S0952836904005011.

Molina GI. 1782. Saggio sulla storia naturale del Chili. Bologna: Stamperia di S. Tommaso d’Aquino.

Moyle RG, Andersen MJ, Oliveros CH, Steinheimer FD, Reddy S. 2012. Phylogeny and biogeography of the core babblers (Aves: Timaliidae). Systematic Biology 61:631–651 DOI 10.1093/sysbio/sys027.

Oliver W, MacKinnon J, Heaney L, Lastica E. 2008. Rusa alfredi. The IUCN red list of threatened species. Version 2014.3. Available at www.iucnredlist.org (accessed on 17 April 2015).

Pääbo S, Poinar H, Serre D, Jaenicke-Després V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M. 2004. Genetic analyses from ancient DNA. Annual Review of Genetics 38:645–679 DOI 10.1146/annurev.genet.37.110801.143214.

Pitra C, Fickel J, Meijaard E, Groves PC. 2004. Evolution and phylogeny of old world deer. Molecular Phylogenetics and Evolution 33:880–895 DOI 10.1016/j.ympev.2004.07.013.

Rafinesque CS. 1817. Descriptions of seven new genera of North American quadrupeds. American Monthly Magazine and Critical Reviews 2(1):44–46.

Rafinesque CS. 1832. Description of some of the fossil teeth in a cave in Pennsylvania. Atlantic Journal 1:109–110.
Randi EJP, Mucci N, Claro-Hergueta F, Bonnet A, Douzery EJP. 2001. A mitochondrial DNA control region phylogeny of the Cervinae: speciation in Cervus and implications for conservation. Animal Conservation 4:1–11 DOI 10.1017/S1367943001001019.

Ronquist F, Teslenko M, Van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Systematic Biology 61:539–542 DOI 10.1093/sysbio/sys029.

Rossi RV. 2000. Taxonomia de Mazama Rafinesque, 1817 do Brasil (Artiodactyla, Cervidae). MSc Thesis, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil.

Rumiz DI, Pardo E. 2010. Chapter 21 Peruvian dwarf brocket deer Mazama chunyi (Hershkovitz 1959). In: Duarte JMB, Gonzalez S, eds. Neotropical cervidology: biology and medicine of Latin American deer. Jaboticabal and Gland: Funep in collaboration with IUCN, 185–189.

Smith WP. 1991. Odocoileus virginianus. Mammalian Species 388:1–13.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22(21):2688–2690 DOI 10.1093/bioinformatics/btl446.

Thomas O. 1908. LVI. A new deer of the Brocket group from Venezuela. Journal of Natural History 1(4):349–350.

Timmins RJ, Steinmetz R, Sagar Baral H, Samba Kumar N, Duckworth JW, Anwarul Islam M, Giman B, Hedges S, Lynam AJ, Fellowes J, Chan BPL, Evans T. 2008. Rusa unicolor. The IUCN red list of threatened species. Version 2014.3. Available at www.iucnredlist.org (accessed on 17 April 2015).

Van Bemmel ACV. 1952. Contribution to the knowledge of the genera Muntiacus and Arctogalidia in the Indo-Australian Archipelago (Mammalia, Cervidae & Viverridae). Beaufortia 16:1–50.

Vogliotti A, Duarte JMP. 2009. Discovery of the first wild population of the small red brocket deer Mazama bororo (Artiodactyla: Cervidae). Mastozoologıa Neotropical 16(2):499–503.

Von Zimmermann EAW. 1778–1783. Geographische Geschichte des Menschen, und der allgemein verbreiteten vierfüßigen Thiere, nebst einer Hieher gehörigen zoologischen Weltcharte. Vol. 2 Geographische Geschichte des Menschen, und der vierfüßigen Thiere. Zweiter Band. Enthält ein vollständiges Verzeichnis aller bekannten Quadrupeden. Vol. 3. Leipzig: Weygandschen Buchhandlung, 1–276.

Voris HK. 2000. Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems and time durations. Journal of Biogeography 27:1153–1167 DOI 10.1046/j.1365-2699.2000.00489.x.

Vislobokova IA. 1980. On systematical position of the deer from Pavlodar and the origin of 850 neocervines. Paleontological Journal 3:91–106.
Wagner JA. 1844. *Die Säugetiere in Abbildungen nach der Natur mit Beschreibungen von Dr. Johann Christian Daniel von Schreber*. Supplementband. Walther, Erlangen 4: i-xii + 1-523 pp.

Webb SD. 2000. Evolutionary history of New World Cervidae. In: Vrba ES, Schaller GB, eds. *Antelopes, deer, and relatives: fossil record, behavioral ecology, systematics, and conservation*. New Haven: Yale University Press, 38–64.

Weber M, González S. 2003. Latin American deer diversity and conservation: a review of status and distribution. *Ecoscience* 10(4):443–454.

Woodruff DS. 2003. Neogene marine transgressions, palaeogeography and biogeographic transitions on the Thai–Malay Peninsula. *Journal of Biogeography* 30:551–567 DOI 10.1046/j.1365-2699.2003.00846.x.