

Molecular phylogeny of the genus *Muntiacus* with special emphasis on the phylogenetic position of *Muntiacus gongshanensis*

DEAR EDITOR,

Muntjac deer (Cervidae: *Muntiacus*) are often cited as an excellent model for the study of vertebrate evolution due to their fast rate of change in chromosome number among vertebrates. However, the phylogenetic relationships within *Muntiacus* generally, and the taxonomic status of *Muntiacus gongshanensis* specifically, remain unclear. Here, the phylogenetic relationships within *Muntiacus* were studied using mitochondrial genome (mitogenome) and cytochrome *b* (cyt *b*) segments. Our results recognize 12 species within *Muntiacus* and support the controversial species *M. gongshanensis*, *M. putaoensis*, and *M. malabaricus*. Furthermore, Bayesian inference (BI) and maximum-likelihood (ML) approaches revealed *M. gongshanensis* and *M. crinifrons* to be closely related species, with *M. feae* as their sister species, and *M. putaoensis* and *M. truongsongensis* to be closely related, with *M. rooseveltorum* as their sister species. The distribution range of *M. gongshanensis* was also confirmed in southwest China (Namdapha, Modong, Zayu and Gongshan) and northern Myanmar (Putao). The results of this study provide insight into the evolution of *Muntiacus* and further provide a molecular basis for the taxonomic evaluation of the genus in the future and fundamental data for the conservation of *M. gongshanensis*.

Species of the genus *Muntiacus* Rafinesque, 1815 (Artiodactyla: Cervidae: Muntiacinae) are distributed throughout Southeast Asia and southern China (Supplementary Figure S1). *Muntiacus* species are of great interest in evolutionary studies because of their chromosomal variations and recent classification of several new species (Wang & Lan, 2000; Yang et al., 1997). Indeed, the number of known muntjac species has increased in the last 30 years,

including *M. gongshanensis*, Ma, Wang & Shi, 1990; *M. vuquangensis* Tuoc, Dung, Dawson, Arctander & Mackinnon, 1994; *M. truongsongensis* Gao, Tuoc, Dung, Wikramanayake, Amato, Arctander & Mackinnon, 1998; and *M. putaoensis* Amato, Egan & Rabinowitz, 1999. Although much attention has been paid to *Muntiacus*, the taxonomy of this genus remains controversial (Supplementary Table S1). There is dispute regarding the number of classified species within *Muntiacus*. Indeed, only five species were reported in 1986 (Ma et al., 1986), but 16 species were listed in 2011 (Groves & Grubb, 2011). Thus, much debate remains regarding the taxonomic status of reported species (Supplementary Table S1). Presently, 13 species of *Muntiacus* are recorded in the IUCN list, 54% of which are considered Data Deficient.

Species represent the basic unit of biodiversity. Ambiguous definitions and diagnoses can impact biodiversity conservation, and potentially result in common species being classified as endangered while truly endangered species are neglected (Hong, 2016). While several extant species of muntjac differ very generally in body size, color, and antlers, many share similar basic morphology that can make them difficult to distinguish. Furthermore, as the distribution ranges of muntjac species continue to be updated, the taxonomic status of certain groups as independent species is questionable (Groves & Grubb, 1982; James et al., 2008; Le et al., 2014; Ma et al., 1990; Smith et al., 2010).

The significance of historical distributions of muntjac species must be interpreted with caution. For example, identification of archaeological materials has indicated that the Holocene distributions of some muntjac species were much more extensive than historical records indicate (Turvey et al., 2016). *Muntiacus gongshanensis*, described in Gongshan

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County, Yunnan Province, southwestern China (Ma et al., 1990), is one of the least-known ungulate species in China (Liu & Wu, 2019), including its taxonomic status and distribution range. Previously, it was considered a northern subspecies of *M. feae* or western subspecies of *M. crinifrons* (Smith et al., 2010). Furthermore, recent reports of *M. crinifrons* in Tibet, China, far from its generally accepted range (eastern China), may result from its misidentification with *M. gongshanensis* described at Mount Gaoligong (Chen et al., 2008; Timmins & Duckworth, 2016). *Muntiacus gongshanensis* is described as Data Deficient by the IUCN but is listed as Critically Endangered in the latest red list of China's vertebrates (Jiang et al., 2016). *Muntiacus crinifrons* is listed as Vulnerable in the latest red list of the IUCN but is categorized as Endangered in the latest red list of China's vertebrates. The current population trends for both *M. gongshanensis* and *M. crinifrons* show a decline (Timmins & Duckworth, 2016). Thus, there is an urgent need to reconstruct the taxonomic classification and phylogeny of the genus *Muntiacus* to establish appropriate levels of protection for muntjac species.

The phylogeny of the genus *Muntiacus* has long been debated (James et al., 2008; Timmins & Duckworth, 2016). Comparative phylogenomic approaches can solve the problem of deep branches resulting from rapid radiations (Chen et al., 2019; Jiang et al., 2019). Therefore, mitogenomes have been used to explore the interspecific relationships within *Muntiacus*. Previous phylogenies of *Muntiacus* have largely been based on morphological characters (Ma et al., 1986) and partial fragments of mitochondrial DNA (Cao et al., 2002; Wang & Lan, 2000; Yi et al., 2002). In recent years, mitogenomes have been used for phylogenetic reconstruction of *Muntiacus* (Li et al., 2017; Martins et al., 2017; Singh et al., 2019; Srisodsuk et al., 2018; Zhang et al., 2019a), but this approach has been limited to one or only a few species. Complete mitogenomes contain increased phylogenetic signals compared to partial fragments and therefore provide more comprehensive insight into the phylogeny of taxa. In the classification of species, phylogenetic relationships based on complete mitochondrial sequences can be used as a reference (Zhang et al., 2019b). In this study, we sequenced and annotated the mitogenome of *M. gongshanensis*, then reconstructed the phylogenetic relationships within the genus by combining our data with 30 complete mitogenomes and an additional 18 *cyt b* sequences representing species of *Muntiacus* from GenBank. We also confirmed the distribution range of *M. gongshanensis*.

Both ML and BI analyses recovered similar tree topologies, and all nodes had significant support, except for *M. putaoensis* with other muntjacs (bootstrap support for ML/posterior probability in BI=73/0.54). Twelve monophyletic clusters were contained within two major clades on the tree (Figure 1). The first major clade included *M. reevesi*, *M. vuquangensis*, *M. rooseveltorum*, *M. truongsongensis*, and *M. putaoensis*. The second major clade included *M. atherodes*, *M. malabaricus*, *M. vaginalis*, *M. muntjak*, *M. feae*, *M. crinifrons*, and *M.*

gongshanensis. In the first major clade, *M. reevesi* differentiated first, followed by *M. vuquangensis*. In the posterior subclade, *M. rooseveltorum* differentiated first, and *M. putaoensis* was most closely related to *M. truongsongensis*. In the second major clade, *M. atherodes* differentiated first, and the remaining species split into two subclades. The '*M. muntjak* clade' included the Sri Lankan red muntjac (*M. malabaricus*), southern red muntjac (*M. muntjak*), and northern red muntjac (*M. vaginalis*). *Muntiacus malabaricus* differentiated first, with *M. muntjak* and *M. vaginalis* differentiating subsequently. *Muntiacus gongshanensis* was in the same clade as *M. crinifrons* and *M. feae*. *Muntiacus feae* differentiated first, followed by *M. crinifrons* and *M. gongshanensis*.

We used network analysis of the *M. gongshanensis*, *M. crinifrons*, and *M. feae* haplotypes to provide further resolution of the closely related *M. gongshanensis* haplotypes and reveal the interspecific relationships of the three species (Figure 1C). Results were similar to those obtained from the phylogenetic tree; the haplotype network supported the recognition of three clusters: *M. crinifrons* and *M. feae* were segregated into two clusters and distinct haplotypes 1 and 2, and also separated from *M. gongshanensis* in 25 and 12 mutational steps, respectively; eleven haplotypes (haplotypes 3 to 13) of *M. gongshanensis* ($n=13$) were shared among three different regions. Network analysis of the *M. gongshanensis* haplotypes showed a star-like appearance. Central haplotype 11 was shared by *M. gongshanensis* individuals from the Namdapha region, China, and Putao, Myanmar. Haplotype 7 was shared by *M. gongshanensis* from Yunnan (China) and Putao (Myanmar). The other haplotypes were shared by the original collection region of each individual (Figure 1C).

The complete mitogenomes of *Muntiacus* species provide a deeper understanding of the phylogenetic relationships within the genus. To date, our results represent the most comprehensive analysis of molecular data for *Muntiacus* and will help to elucidate the evolutionary relationships within the genus. There is no question of the validity of *M. reevesi* as an independent species (Figure 1). Our results support *M. putaoensis*, *M. vuquangensis*, *M. truongsongensis*, and *M. rooseveltorum* being of the same lineage. *Muntiacus putaoensis* is the most recently discovered species of muntjac, as confirmed based on partial fragments of mtDNA (Amato et al., 1999). Subsequent studies suggest that *M. putaoensis*, *M. truongsongensis*, and *M. rooseveltorum* likely belong to the *M. rooseveltorum* species complex (James et al., 2008; Li et al., 2017). Our results support *M. putaoensis*, *M. truongsongensis*, and *M. rooseveltorum* as independent species, with *M. putaoensis* most closely related to *M. truongsongensis* and *M. rooseveltorum* as their sister species. Notably, the geographical range of *M. vuquangensis* overlaps with that of *M. rooseveltorum* and *M. truongsongensis* in Laos and Vietnam, but *M. putaoensis* does not overlap with *M. rooseveltorum* or *M. truongsongensis*, despite being more closely related to these two species (Figure 1B).

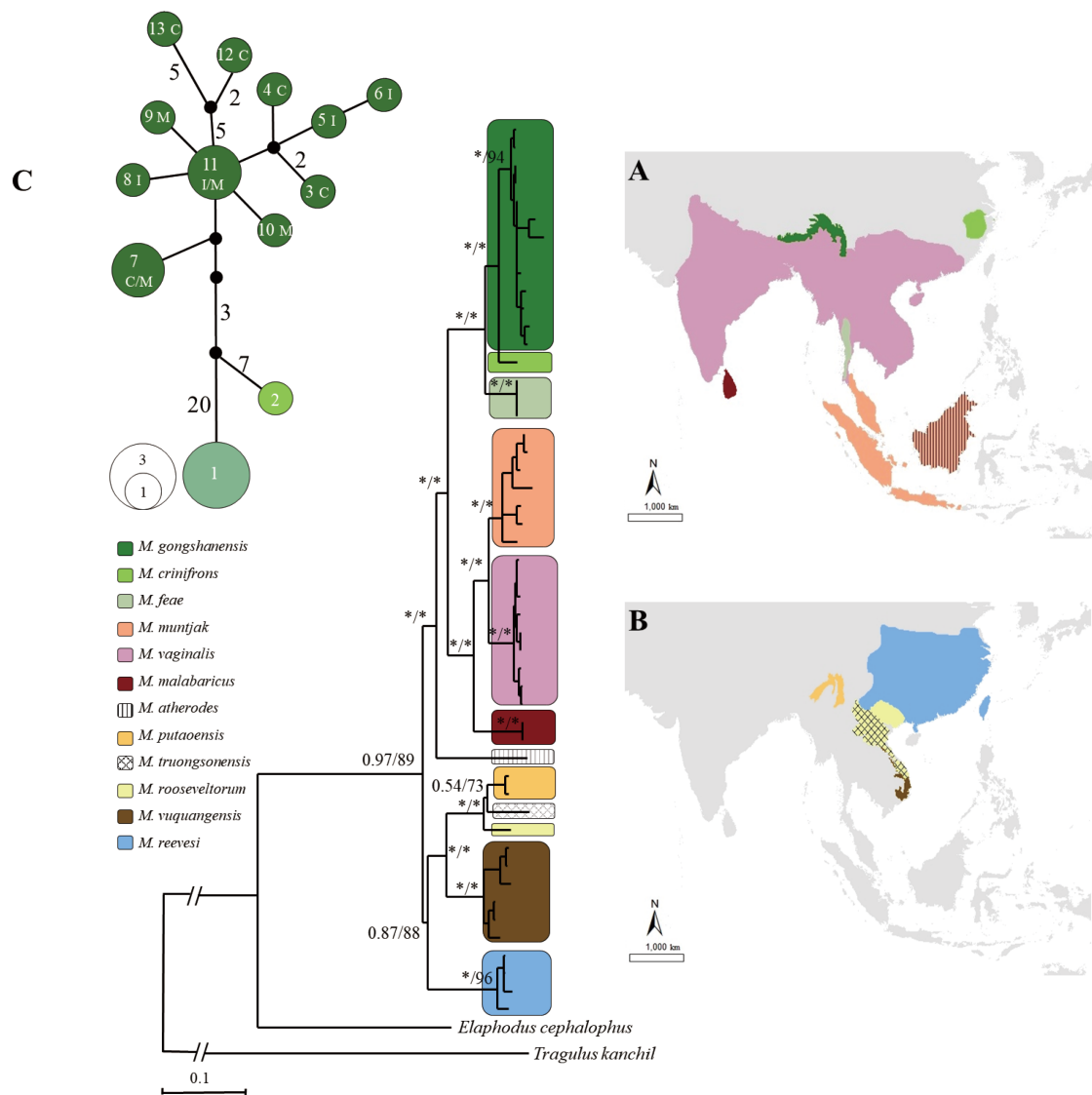


Figure 1 Bayesian inference (BI)- and maximum-likelihood (ML)-based phylogenetic trees for 12 muntjac species based on mitochondrial DNA

Numbers on clades indicate posterior probability for BI followed by bootstrap support for ML analyses of the node. Asterisks indicate values of 100 (ML) and 1.00 (BI). Different species are represented by specific colors, with their distribution ranges represented by corresponding colored areas on the map. A, B: Show distribution of *Muntiacus* genus; C: Median-joining network of *cyt b* gene fragments of *M. feae*, *M. crinifrons*, and *M. gongshanensis*. Haplotype numbers correspond to those in Supplementary Table S2. Circle size is proportional to haplotype frequency, with scale provided in lower left corner; fill color denotes different species. Lines represent one mutational step, except when indicated otherwise by numbers; black circles represent missing vectors.

At present, the classification of red muntjacs is still controversial. Although up to five species have been described, the most commonly accepted are the northern red muntjac (*M. vaginalis*) and southern red muntjac (*M. muntjak*) (Groves & Grubb, 2011). Recent research supports the delineation of either three monotypic species or three subspecies of red muntjac (Martins et al., 2017). Our research supports the classification of red muntjac into three monotypic species. We propose *M. malabaricus* as a valid monotypic

species of *Muntiacus* because our phylogenetic trees strongly suggest that *M. malabaricus* is monophyletic; notably, *M. malabaricus* also differs morphologically from its most closely related species, *M. vaginalis*, being smaller in size with shorter antlers and pelage color differentiation (Groves & Grubb, 2011). In addition, *M. malabaricus* and *M. vaginalis* are distributed relatively independently. Our phylogenetic analyses indicate that subspecies or multiple populations may exist within the *M. muntjak* and *M. vaginalis* species.

Our results also suggest that *M. gongshanensis* is a valid species. We base this conclusion on the following evidence: (a) Our phylogenetic trees strongly support *M. gongshanensis* and *M. crinifrons* as monophyletic; (b) The haplotype networks strongly support the separation of *M. gongshanensis* and *M. crinifrons* by 12 mutational steps and three missing vectors; (c) The geographical distributions of *M. gongshanensis* and *M. crinifrons* do not overlap at all (Figure 1A), being in the west and east of China, respectively, and separated by more than 2 000 km and many mountain and river barriers; (d) *Muntiacus gongshanensis* shows different morphology from *M. crinifrons*, e.g., *M. gongshanensis* is small, light in color, long in hoof, and short in tail, with no crown (Groves & Grubb 2011; Ma et al., 1990). In addition, although *M. gongshanensis* possesses the same number of karyotypes as *M. crinifrons*, they are different in structure (Shi & Ma, 1988). However, results showed that *M. gongshanensis* is most closely related to *M. crinifrons*, followed by *M. feae*, similar to previous studies (Ma et al., 1986; Wang & Lan, 2000; Zhang et al., 2019a).

Some researchers have argued that *M. gongshanensis* is endemic to China (Jiang et al., 2016; Wang, 2003). However, other studies have shown that the distribution of *M. gongshanensis* may have extended to northern Myanmar and India (Smith et al., 2010; Timmins & Duckworth, 2016). Our study provides molecular evidence of *M. gongshanensis* distribution in southwest China (including Namdapha region), northern Myanmar. Eight sequences published as *M. crinifrons* are in fact *M. gongshanensis* (GenBank accession Nos.: DQ445732–DQ445735, EF523661–EF523664). These samples were obtained from Tibet and the Namdapha region, respectively (Chen et al., 2008; James et al., 2008). In addition, images of *M. gongshanensis* were acquired in the Tengchong Section of Gaoligongshan National Nature Reserve, Yunnan Province in May 2018 (Huang et al., 2019). In summary, *M. gongshanensis* is distributed in southwest China (Namdapha, Modong, Zeyu, and Gongshan), northern Myanmar (Putao). The expansion of the *M. gongshanensis* distribution provides new evidence for its assessment.

Although *Muntiacus* is a model genus for studying evolution, research remains scarce. At present, 54% of *Muntiacus* species listed in the IUCN have not been assessed due to insufficient data, although evidence indicates that their populations are declining. Therefore, we strongly recommend the planning of effective conservation strategies for *Muntiacus* and for these distant regions to maintain genetic diversity and protect these precious species. This study improves our understanding of the phylogenetic relationships within the *Muntiacus* genus and will assist in future research and the protection of genetic diversity of muntjacs.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.C.Z., G.G.L., and R.C.Q. conceived, designed, and performed the experiments and analyzed the data. Y.H.L., R.L., and K.M. performed field and laboratory work. Y.C.Z. analyzed the data. Y.C.Z. and G.G.L. participated in writing the manuscript. All authors read and approved the final version of the manuscript.

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