



## Flightless *Notaris* (Coleoptera: Curculionidae: Brachycerinae: Eriirhinini) in Southwest China: monophyly, mtDNA phylogeography and evolution of habitat associations

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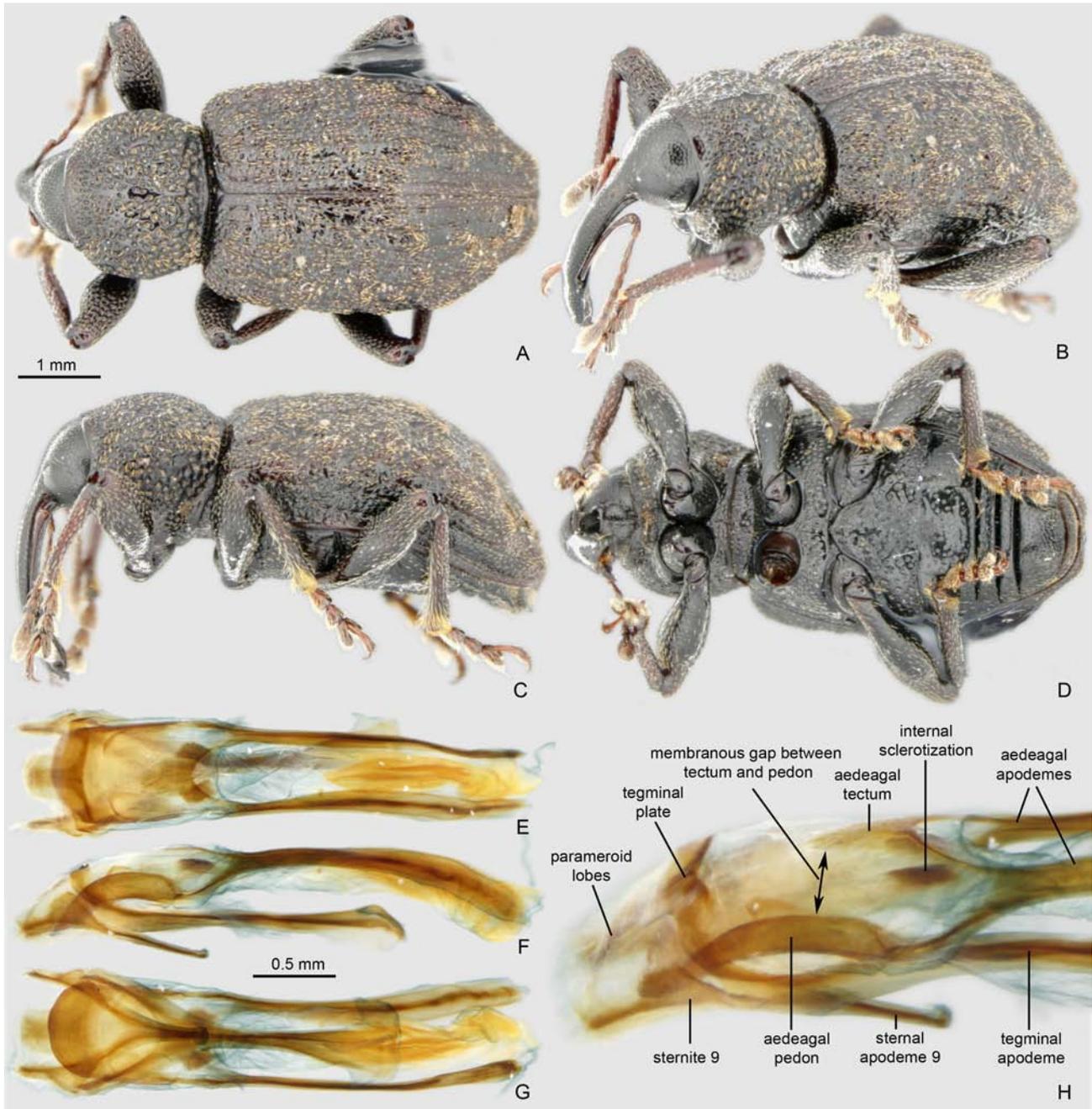
### Abstract

This paper reports the recent discovery of flightless populations of weevils of the genus *Notaris* in Yunnan and Sichuan provinces of China. Specimens were found in the middle or high altitude mountains (2440–4195 m), by either sifting leaf litter in the deciduous forest and among alpine *Rhododendron* shrubs, or by turning rocks in the alpine zone. These finds extend southwards the Asian range of this Holarctic genus and report its highest altitudinal records. DNA barcodes of 127 specimens were phylogenetically analysed, of them 42 are those of newly discovered *Notaris* from Southwest China. The genera *Notaris* and *Tournotaris* consistently formed a clade, with *Tournotaris* nested inside *Notaris* in Maximum Parsimony (MP) and Maximum Likelihood (ML) analysis. The newly discovered flightless *Notaris* from Southwest China were either monophyletic (MP) or paraphyletic with respect to volant Holarctic *N. aethiops* (ML); the latter placement being likely an artefact. A strict linear molecular clock approach suggests a pre-Pliocene separation of *Notaris* populations in Southwest China. Habitat associations of these high-altitude flightless *Notaris* contrast sharply with that of the predominantly volant lowland riparian *Notaris* and other Eriirhinini. We hypothesise that evolution of habitat selection in *Notaris* went from lowland riparian, to high altitude (via uplift of the Tibetan Plateau and adjacent regions of Central Asia), and then to forest leaf litter (via subsequent erosions of isolated mountains such as Emei Shan in Sichuan losing the alpine zone and forcing *Notaris* into the forest floor). Taxonomic uncertainty of Asian *Notaris* is addressed and remains unresolved due to uninformative morphology and conflicting DNA signal. Identities of two obscure and likely closely related species, *Notaroides brevirostris* and *Notaris kozlovi* from nearby SE Qinghai and NW Sichuan, respectively, are discussed and illustrated. Pending further research, all reported flightless *Notaris* from Yunnan and Sichuan are hypothesised to form a clade, for which the available name *N. kozlovi* is used. Habitus and genitalia of *Notaris* specimens from the newly detected populations are illustrated.

**Key words:** CO1, DNA barcoding, Hengduan mountains, Tibetan Plateau, *Tournotaris*, *Notaroides*, weevils

### Introduction

This project saw its inception on June 15, 2010, when a series of a relatively large weevil (Fig. 1) somewhat resembling *Kurilio monachum* Zherichin et Egorov (Grebennikov 2014b, figs 14 G,E), was detected in two sifted leaf litter samples taken in the deciduous forest on the slope of Mount Emei in Sichuan, China (Figs 9, 10A). Three days later another sample which was taken in similar circumstances just 1.4 km away contained somewhat similar specimen (Fig. 2A). The following year, 2011, a series of smaller and likely congeneric specimens (Fig. 2B) was similarly sifted from the forest litter in the region of Mount Gongga, some 120 km W of Mount Emei. In 2012 similar beetles were sifted from the litter in vicinity of Songpan, Sichuan (Figs 2C, 3C) and abundantly found by turning stones in the alpine zone of Mount Haba, Yunnan, at relatively high altitude above 4000 m (Figs 3A–B). Besides *Kurilio*, the external appearance of the newly detected specimens resembled species of the Holarctic genus *Notaris* Germar. This hypothesis was challenged by the fact that neither forest leaf litter nor the alpine zone was known as the preferred *Notaris* habitat, which are predominantly riparian species inhabiting much lower altitudes.



**FIGURE 1.** *Notaris kozlovi* from Mount Emei, Sichuan, V4192. A–D: habitus; E–G: aedeagus, tegmen and abdominal sternite 9, ventral (E), right lateral (F), dorsal (G) and right ventro-lateral (H, enlarged).

Furthermore, effaced elytral shoulders of the newly collected beetles suggested brachyptery and flightlessness very rarely recorded in *Notaris* and its Eirrhiniini relatives. Additionally, no *Notaris* species have been known to occur so deep inside the Southwest China highlands. Overall, these beetles seemed novel and posed an interesting evolutionary puzzle.

Subsequent closer comparison of the newly discovered Yunnan and Sichuan specimens among themselves and with those of *Kurilio* and *Notaris*, aided by colleagues' consultations (see Acknowledgements), analysing DNA barcodes and detection of their pedotectal male genitalia (= "primitive", dorsal tectum and ventral pedon distinctly separated by a lateral membrane; Fig. 1; Alonso-Zarazaga 2007) gradually corroborated their affinities with *Notaris*. This, in turn, triggered further efforts to place them in the non-existent phylogenetic framework of the genus and to apply Linnaean nomenclature.

The Holarctic genus *Notaris* includes about twenty predominantly winged species widely distributed throughout temperate North America, all of Europe and most of temperate lowland Asia, including most of Russia,

Kazakhstan, Mongolia and Japan (Caldara 2011). Except for the widely distributed lowland rice pest *N. oryzae* Ishida recently recorded as far south as Laos (Kojima *et al.* 2010), only two more *Notaris* species were cited in the Catalogue of Palaearctic Coleoptera for the whole of China: *N. distans* Faust from Xizang (=Xinjiang Uyghur Autonomous Region) and *N. mandshurica* Voss from Heilongjiang (Caldara 2011); both regions forming the NW and NE extremities of China, respectively. A handful of poorly known *Notaris* species, however, were described from the highlands of Central Asia (Kyrgyzstan, Kazakhstan, Tajikistan, Uzbekistan), Mongolia and West Siberia (see Caldara 2011), which, therefore, suggests the possible presence of the genus farther south. One highly obscure *Notaris* species omitted in the Catalogue has indeed been relatively recently described from hundred year old specimens collected in the extreme north-east corner of the Sichuan province (*N. kozlovi* Korotyaev, Fig. 4; only about 500 km NNW from Mount Gongga, Fig. 9), along with the nearly sympatric and likely related *Notaroides brevirostris* Korotyaev attributed to a genus of its own and only known from the holotype. All these considerations indicated that the genus *Notaris* might indeed accommodate the newly discovered Chinese species, although not before a number of obscure phylogenetic and neglected taxonomic issues get resolved.

The goal of the present paper is to report morphological, biological and genetic diversity of the newly detected *Notaris* in Southwest China. This paper documents our attempts to variously test monophyly of the genus and that of the newly detected flightless populations in China and to place them in the newly developed phylogenetic framework of *Notaris* and its allies. Using our phylogenetic results, we hypothesize the direction of evolutionary changes in morphology, biology and distribution of Chinese *Notaris* within the temporal framework of orographic events using linear molecular clock approach. Our strenuous attempts to generate species hypotheses for the newly discovered Yunnan and Sichuan populations were, however, inconclusive. This was due to inadequate and conflicting information available from four independent data sources (morphology, mtDNA, biology, distribution), which could not be coherently balanced by proposing reliable species boundaries. Existence of two names available for two poorly known although likely most closely related organisms from the closely adjacent territories (*Notaris kozlovi* and *Notaroides brevirostris*) added further taxonomic uncertainty. Overall this paper represents our attempt to find a compromise between the necessity to report and interpret new evolutionary data for a group of poorly known organisms, and the limits imposed by the lack of data.

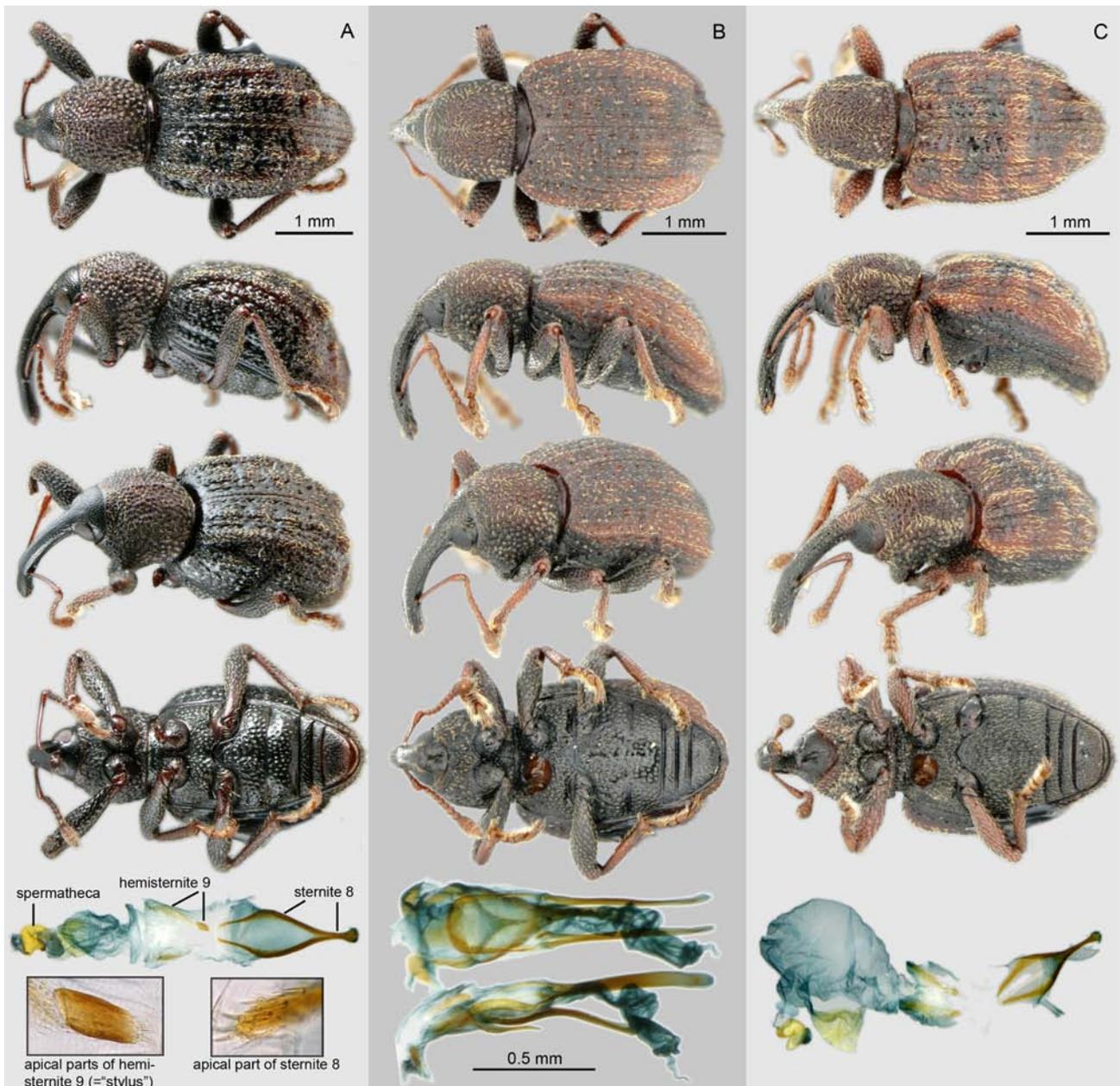
## Material and methods

Museum codes, followed by the name of the curator:

CNC Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Canada (P. Bouchard);  
SNSB Zoologische Staatssammlung Muenchen, Munich, Germany (M. Balke);  
ZIN Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (B.A. Korotyaev).

Concepts of the Curculionoidea family-group names follow those of Bouchard *et al.* (2011) with subsequent modifications of Oberprieler (2014), who downgraded the former family Brachyceridae to subfamily status within Curculionidae and significantly changed delimitations of its six tribes, as compared to their older concepts by Alonso-Zarazaga & Lyal (1999). The chronostratigraphic timing is that of Cohen *et al.* (2013) with the Pliocene-Pleistocene boundary set up at 2.588 Ma. Male genitalia nomenclature (Fig. 1) follows that of Wanat (2007). Map on Fig. 9 was generated using the online SimpleMapper tool (Shorthouse 2010).

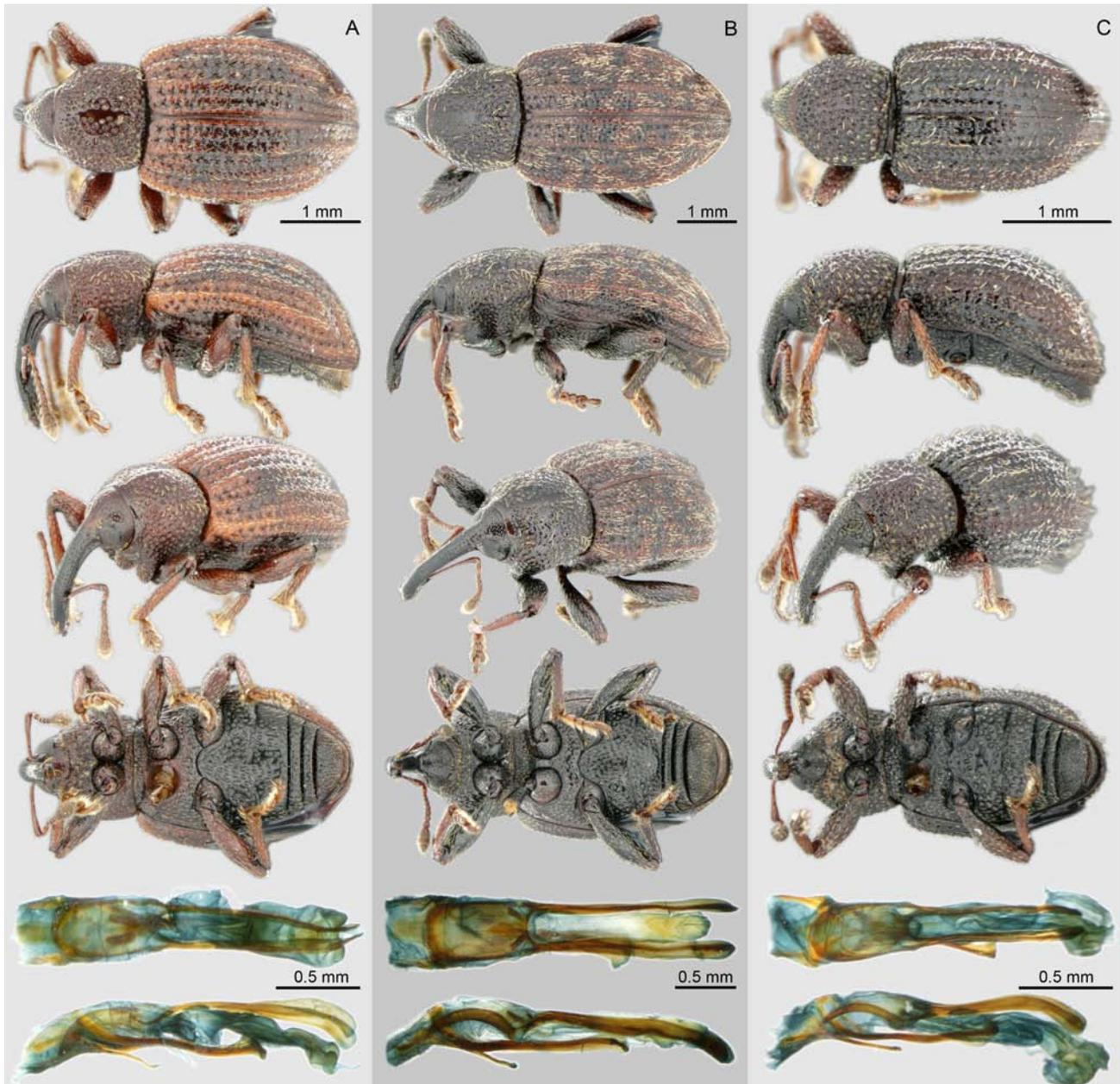
**Analysis design.** The analysis implemented in this paper was designed to test the following hypotheses: 1. monophyly of all, and each among, the newly discovered *Notaris* from Southwest China, their affinities with other *Notaris*, and monophyly of the genus, particularly with respect to *Tournotaris* Alonso-Zarazaga & Lyal; 2. single origin of flightlessness in *Notaris*; and 3. direction of evolutionary changes in Erihrinini habitat associations by ordering three states: (a.) lowland riparian, (b.) mid-altitude forest litter and (c.) high altitude alpine under stones. For these purposes we DNA barcoded 42 specimens (of the total 93 collected) from each geographic location supporting populations of *Notaris* newly discovered in Yunnan and Sichuan. These 42 sequences formed the in-group of the analysis. The near outgroup included other *Notaris* and *Tournotaris* with available DNA barcode data,



**FIGURE 2.** *Notaris kozlovi* from Southwest China. A: V1207 from Mount Emei, Sichuan; B: V2545 from Mount Gongga, Sichuan; C: V5376 from Songpan, Sichuan.

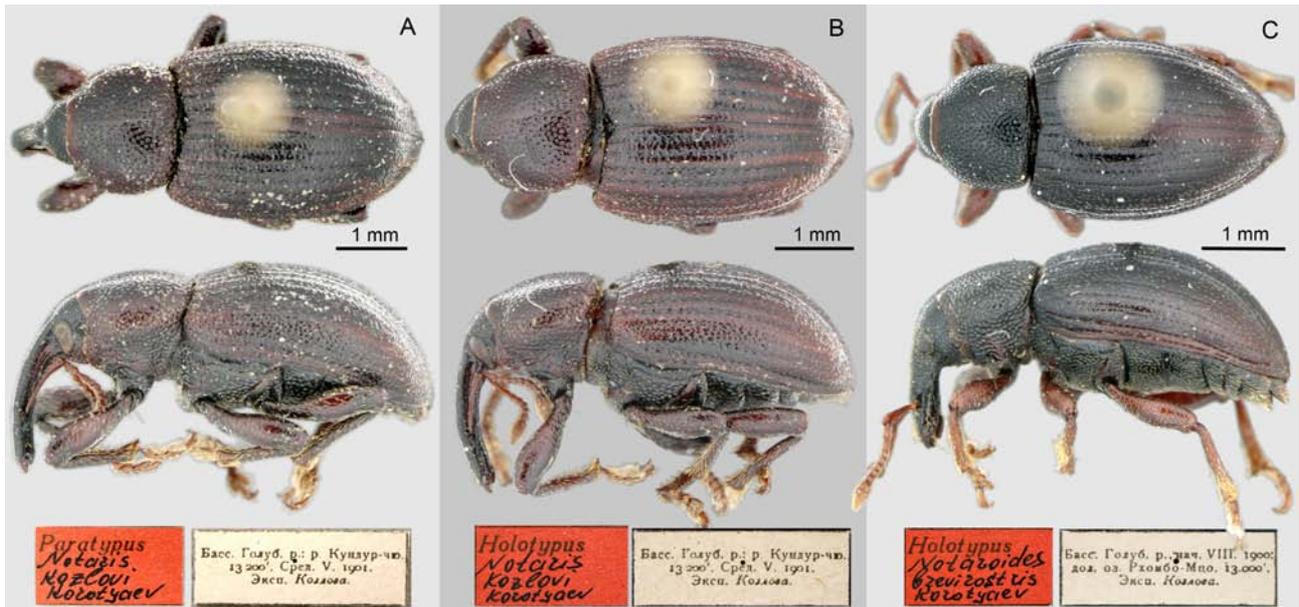
all of them winged and volant, except for 10 specimens of an unidentified *Notaris* from the alpine zone of Tran-Ili Alatau (=mountain range) in southern Kazakhstan (Figs 5, 9). The distant outgroup includes representatives of Curculionidae, mainly of Brachycerinae and, particularly, Eirrhiniini (Fig. 6). All topologies were rooted on a single representative of the distantly related family Anthribidae.

**Original matrix composition.** The original DNA barcode matrix consisted of 127 terminals and 658 aligned positions. None of the sequences was shorter than 407bp, while 116 (91%) sequences were longer than 600bp. DNA barcodes were assembled from three sources. Majority (80) were prepared by us from specimens mainly collected by us in China, Kazakhstan or elsewhere, although 19 of them were generated from European Eirrhiniini specimens sent to us by Marek Wanat. These 80 specimens are stored in CNC and have a unique identifier label with the code CNCCOLVG0000XXXX, which is shortened to VXXXX when specimens are cited in the text below or on the tree (Figs 6, 7). Twenty four other sequences (GenBank accessions KJ672278–KJ672301) are those of three winged *Notaris* and *Tournotaris* specimens, plus 21 other Nearctic and Neotropical Brachycerinae (Fig. 6). These specimens are also stored in CNC and have a unique identifier labels with the code CNC COLEO 00XXXX



**FIGURE 3.** *Notaris kozlovi* from Southwest China. A: V4576 from Mount Haba, Yunnan; B: V6193 from Mount Haba, Yunnan; C: V5377 from Songpan, Sichuan.

XX labels, which is shortened to PXXXXXX (Fig. 6). The remaining 23 sequences are those of Hendrich *et al.* (2015) (HQ948166, HQ948231, HQ953327, HQ953434, JF889498, KM440000, KM440104, KM441836, KM442880, KM442926, KM443047, KM444044, KM444501, KM444970, KM446529, KM446840, KM447368, KM447746, KM449076, KM450170, KM450175, KM450893, KM451384) of five European Brachycerinae species (*Grypus equiseti* (F.), *Notaris acridulus* (L.), *N. scirpi* (F.), *Tanysphyrus lemnae* (Paykull), *Tournotaris bimaculata* (F.)); the latter is consistently given in their paper and in BOLD as “*Notaris bimaculatus*” and was changed to *Tournotaris bimaculata* on our trees); their vouchers are stored in SNSB and were not studied by us. All these sequences are available from GenBank under accession numbers indicated on the phylogram (Figs 6, 7). Images of all 127 DNA barcoded specimen, their geographical data, the lab processes, primers, sequences, original electropherograms and other relevant data can be seen online in the publicly accessible dataset “wingless Asian *Notaris*” on the Barcode of Life Database portal (doi: dx.doi.org/10.5883/DS-NOTARIS).



**FIGURE 4.** Type specimens and their labels of two nominative Eirrhini from Southwest China. A–B: *Notaris kozlovi*, paratype (A) and holotype (B); C: *Notaroides brevis*, holotype.

**Five DNA analyses.** The generalized time-reversible model with gamma distributed rate heterogeneity and inferred proportion of invariable sites (GTR+G+I) was used for all model-dependant analyses (A1, A3, A5; see below). **Analysis 1 (A1)** was performed in MEGA6 and utilized the complete matrix of 127 sequences, the Maximum Likelihood (ML) approach, and testing of statistical branch support by 500 bootstrap repetitions. Its resulting topology (Figs 6, 7) is chosen to serve as the most inclusive source of phylogenetic information utilizing all available data and the least amount of assumptions. **Analysis 2 (A2)** was made with all parameters similar to A1, except that instead of ML, the Maximum Parsimony (MP) approach was used. The main purpose of A2 was to test consistency of clades and their statistical support between ML and MP. **Analysis 3 (A3)** and **analysis 4 (A4)** were designed after finding out that all Chinese specimens in A1 and A2 were either paraphyletic with respect to a single representative of winged Holarctic *Notaris aethiops* (F.) from Canada (A1, Fig. 7) or monophyletic (A2). It was, therefore, hypothesized that the placement of the latter species inside the Chinese clade is likely an artefact. To test it, analyses A3 and A4 were performed with all parameters similar to those in A1 and A2, respectively, except that the single sequence of *N. aethiops* was removed from the matrix. **Analysis 5 (A5)** was designed after finding that *Notaris* and *Tournotaris* on one hand, and Chinese *Notaris* on another, were consistently recovered as clades in A1–A4 (Fig. 6). It was, therefore, hypothesized, that they both clades have a unique evolutionary history, which can be dated by using molecular clock approach. In the absence of informative fossil records, the strict linear molecular clock approach was implemented with the nucleotide substitution rate of 0.018 substitutions per site per million year (Myr) per lineage (subs/s/Myr/l) (Papadopoulou *et al.* 2010), which means the divergence rate of 0.036 subs/s/Myr/l. The BEAST v1.8.0 software package (Drummond *et al.* 2012) was used utilizing the original 127 terminal matrix restricted to include only 82 sequences of *Notaris* (including *N. aethiops*) and *Tournotaris*. Additional assumptions were implemented in A5: no burn-in, all flightless *Notaris* of Southwest China forced as monophyletic, and all program parameters kept at their default, including the number of generations (10M). Topologies from all analyses were visualized in FigTree v1.4 (Rambaut 2014).



**FIGURE 5.** Flightless *Notaris* sp. from Kazakhstan, V6165.

## Results

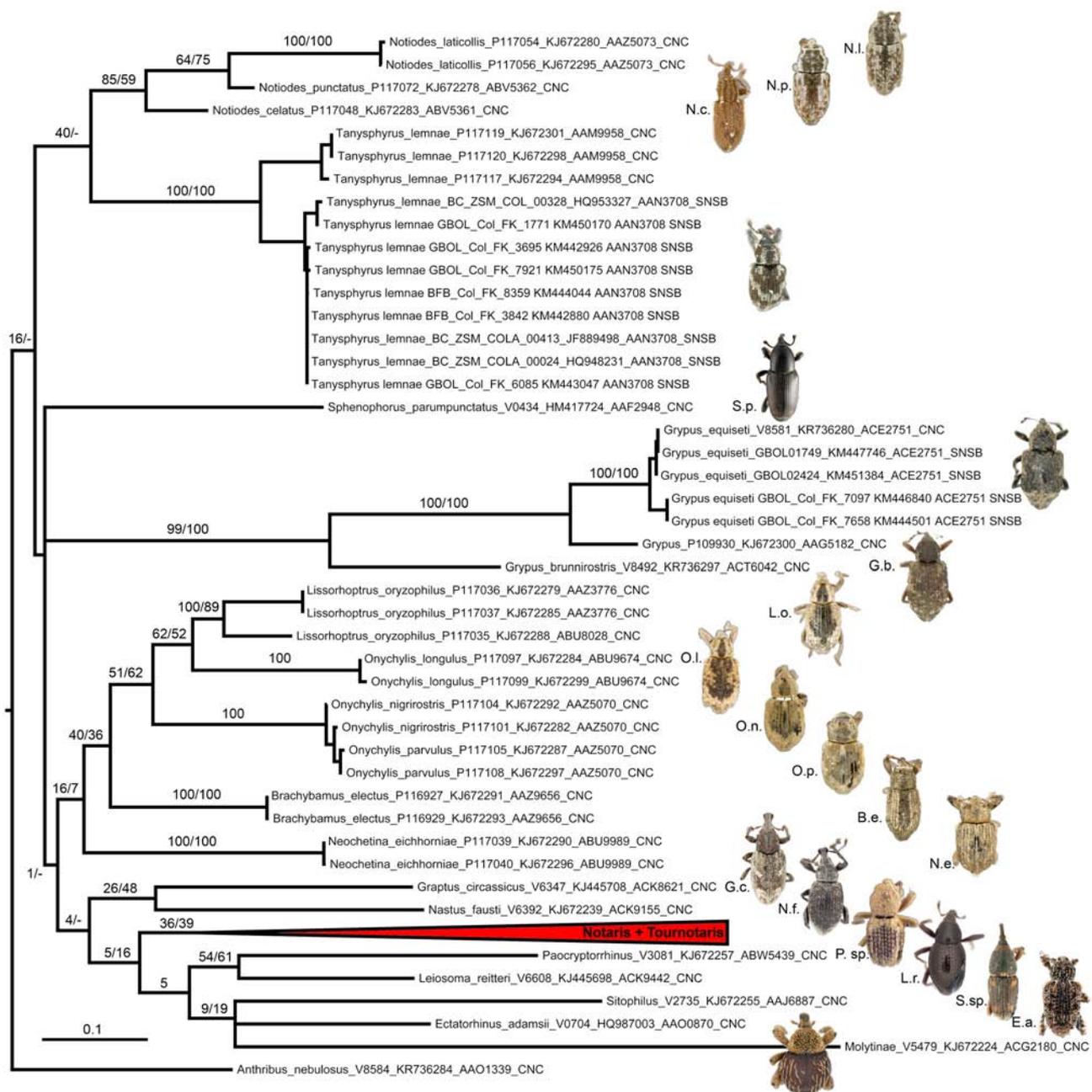
The best ML tree (Figs 6, 7) obtained in Analysis 1 had the highest log likelihood of -11526.3060. The single MP tree (not shown) was 2795 steps long, with a consistency index of 0.221 and a retention index of 0.808. Both analyses recovered a weakly supported clade of *Notaris* + *Tournotaris* with bootstrap support 36% and 39%, respectively. All flightless *Notaris* from Southwest China were recovered as a clade with either inclusion of a single representative of winged Holarctic *Notaris aethiops* from Canada (**A1**, Fig. 7) or without it (**A2**); bootstrap support was 8% and 17%, respectively. Ten likely conspecific sequences of flightless *Notaris* sp. from Kazakhstan were recovered as an absolutely supported clade not most closely related to flightless *Notaris* from Southwest China (Fig. 7). Analysis 3 and 4 recovered topologies and branch support similar to those obtained in A1 and A2, respectively, with the notable exception that A3 bootstrap support for *Notaris* of Southwest China increased from 8% to 23% (Fig. 7).

Temporal analysis in BEAST (Fig. 8) recovered the origin of *Notaris* + *Tournotaris* clade at about 17.7 Ma, that of Chinese flightless *Notaris* clade at about 12.4 Ma, while Kazakhstani flightless *Notaris* separated from its volant relatives at about 12.1 Ma. On the other hand sympatric lineages of mtDNA of *Notaris* on Mount Haba separated at about 5.0 Ma, while those of parapatric *Notaris* on Mount Gongga did so at about 4.0 Ma.

## Discussion

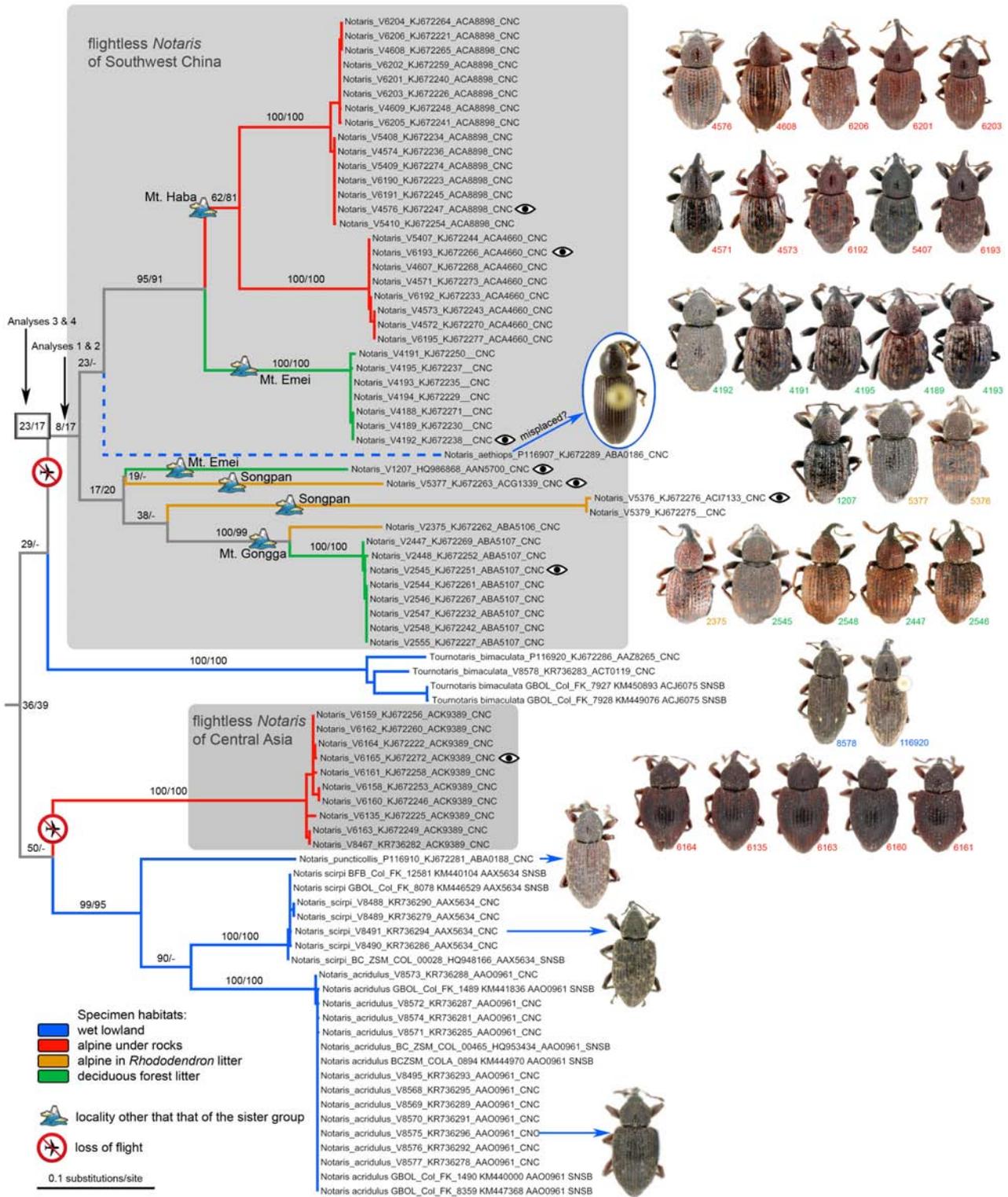
### Monophyly of *Notaris* + *Tournotaris*

Consistent recovery on the clade comprising *Notaris* + *Tournotaris* clade is one of two most significant “positive” results of the present study (or, rather, not negative, following Popper’s logic, 1959). The genus *Notaris* (see the prolonged *Notaris* versus *Eriirhinus* Schoenherr taxonomic confusion resolved by Thompson 2005) has been phylogenetically neglected, similarly to all of many other Eriirhinini (sensu Oberprieler 2014) genera. Seventeen species-group taxa are listed for the Palaearctic Region (Caldara 2011), although at least one more species (*kozlovi*), has been overlooked and another one, *Notaroides brevirostris*, might later be demonstrated as belonging to *Notaris* (see below). The Nearctic Region shares with the Palaearctic one species (*aethiops*) and has a species of its own (*N. puncticollis* (LeConte)). Three other species listed as North American *Notaris* in O’Brien & Anderson (1996: *bimaculata* Fabricius, *flavipilosa* Chittenden, and *goliath* Buchanan) are currently assigned, together with the Palaearctic *ochotica* Korotyaev and *granulipennis* Tournier, to a closely related genus *Tournotaris* (its type species *bimaculata* being Holarctic in distribution; see Thompson 2005). Both genera are decidedly northern temperate in distribution, with just a single pest species found outside the Holarctic region (see Introduction). Among the total of 19 *Notaris* species-group taxa, only three were described during the last hundred years: *N. mandschurica* Voss, *N. kozlovi* and *N. oberti altaica* (Legalov). Overall this genus is a sobering example when a multitude of names remain stable for many decades not because they are well understood and phylogenetically sound, but rather because nobody was willing to critically revise them.



**FIGURE 6.** Maximum Likelihood inference phylogram from Analysis 1 recovering a clade of *Notaris + Tournotaris*. Terminal labels consist of a taxonomic name (to a genus and/or species), followed by BOLD Sample ID, then by GenBank accession, then by Barcode Index Numbers (BINs, Ratnasingham & Hebert 2013), then by Museum abbreviation. Digits at internodes are bootstrap values from Analysis 1 followed, after a slash, by those from Analysis 2. Habitus images (not to scale) are denoted by abbreviated genus and species letters on the same level with the terminal.

The genus *Notaris* has not been the subject of a taxonomic or phylogenetic treatment and, therefore, its monophyly has never been demonstrated. About half a dozen of Central Asian and Siberian species attributed to *Notaris* (many of them described by Johannes K.E. Faust; see Caldara 2011) are inadequately known and cannot be identified to species or perhaps even to genus without such a revisionary work as the study of the type series (an example of these species is *Notaris* sp. V6165 from Kazakhstan; see below). Identifications of adult beetles to the genus *Notaris* should be possible by using keys in Bajtenov (1974), Thompson (2005) and those referenced in the latter. Taxonomic assignment of the newly discovered flightless Chinese specimens to the genus *Notaris*, the latter being an entity of questionable identity, was done mainly on account of their external similarity to other species attributed to *Notaris*, including its type species *Curculio acridulus* L. (fixed by subsequent designation, Westwood 1838),



**FIGURE 7.** Same as Fig. 6, with only the *Notaris* + *Tournaris* clade shown. Placement of volant Holarctic *N. aethiops* shown by a dotted line inside the clade of flightless *Notaris* of Southwest China is likely an artefact; this terminal is not considered as a member of the clade and, therefore, the Analysis 2 bootstrap support of 17 is indicated (together with those of Analyses 3 and 4, boxed). Four different habitats are colour coded. Multiple sequenced specimen habitus images were randomly selected to illustrate morphological variation; terminals marked with an eye symbol are illustrated in four views and their genitalia dissected (Figs 1–3, 5).

coupled with their grouping on the mtDNA-based trees into the *Notaris* + *Tournaris* clade (Fig. 6). The newly detected Chinese populations were not assigned to *Tournaris* because the male abdominal sternite 8 exhibits a long and non-*Tournaris* type “median column” (Thompson 2005, figs 5–8) resembling that of *Notaris* (or

perhaps even that of *Thryogenes festucae* Herbst, see Thompson 2005, fig. 7; the latter genus was not included in the present study). These considerations leave *Notaris* as the most suitable genus to accommodate the newly reported beetles.

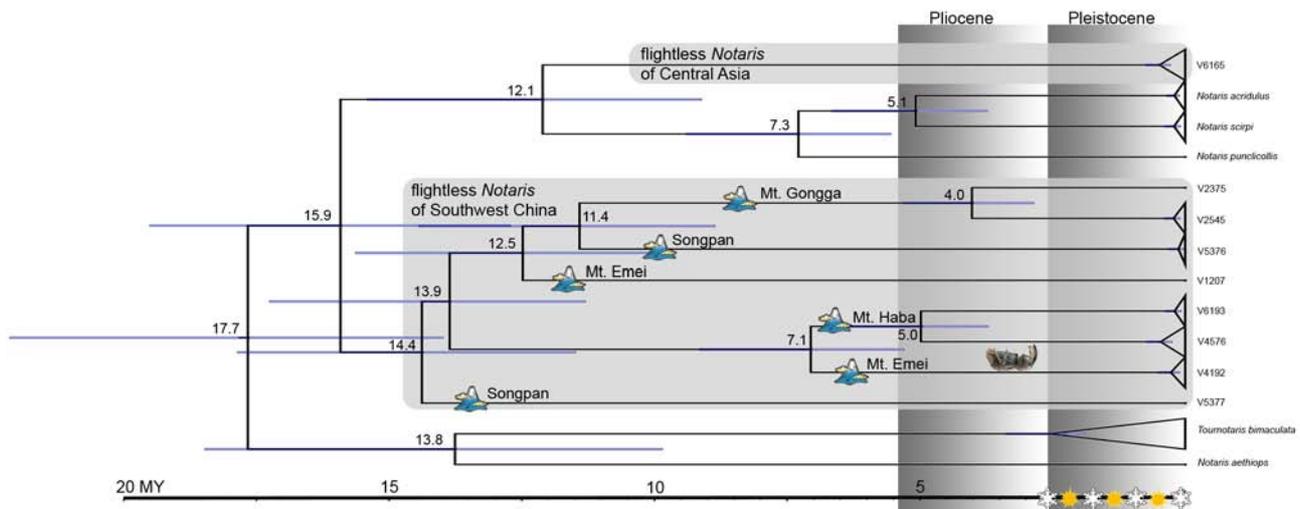
The great majority of non-monotypic invertebrate genus- and family-group taxa continue their taxonomic existence mainly, if not solely, due to the historical reasons of having been originally established for more or less distinguishable (=diagnosable) groups of species and descending till present without an attempt of cladistic justification. The tribe Eirrhiniini, with the genus *Notaris* being its type genus, is not an exception. Thompson (1992), referring to an unpublished manuscript of Guillermo Kuschel, restricted this vaguely defined weevil group to its modern sense as weevils having (a) “orthocerous” male genitalia (Fig. 1H); (b) rostrum-to-head junction in lateral view with the dorsal outline continuously curved and the ventral one strongly angled (Fig. 1C); (c) geniculate antennae; (d) no “pygidium” (=tergite 9) and (e) exposed prementum. The tribal limits within Brachycerinae, the weevil subfamily accommodating *Notaris*, have recently been drastically re-arranged (Oberprieler 2014). Indeed, the previously recognized tribes (Alonso-Zarazaga & Lyal 1999) of former “Eirrhiniinae” included 94 genera, with 15 *incertae sedis* and the rest attributed to seven tribes: Aonychini (1 genus), Arthrostenini (9), Eirrhiniini (38), Himastlophallini (1), Stenopelmini (26), Tadiini (1) and Tanysphyrini (3). Neither the tribes nor the genera have ever been phylogenetically tested, while the subfamily was only marginally included into the much broader phylogenetic attempts (i.e. McKenna *et al.* 2009), some compromised by relying on outdated taxonomy for outgroup choices (Li *et al.* 2007). It should be, therefore, clearly understood that some, if not the majority among the Brachycerinae genera and perhaps even tribes, as well as the subfamily itself, might eventually be demonstrated as not monophyletic, i.e. not existing in the phylogenetic framework.

### Identity of *Notaris kozlovi* and *Notaroides brevirostris*

Before discussing *Notaris* from Southwest China, identity needs to be established of an obscure generic and two equally obscure species names of weevils inhabiting Southwest China and likely most closely related to our in-group: *Notaris kozlovi* and *Notaroides brevirostris*, the latter forming a genus of its own. They were taxonomically validated in Russian, without illustrations, by Korotyaev (1979) based on historical specimens collected during Pyotr Kozlov (1863–1935) expedition. This was Kozlov’s fourth Central Asian expedition (1899–1901) among six he made (and the first among those last three expeditions he headed). The genus *Notaroides* Korotyaev and its type species have been mentioned at least twice in catalogues (Alonso-Zarazaga and Lyal 1999, Caldara 2011), but remains absent in Zoological Record (searched on January 13, 2016). The other name apparently has never been mentioned in any source other than the original description. Both names represent beetles geographically adjacent to our in-group specimens (Fig. 9), possibly closely related to them, and perhaps even conspecific. It is, therefore, necessary to assess identity of beetles signified under these obscure names by using all available sources of information, which are the original descriptions and the study of the type specimens.

The original description of *N. kozlovi* did not specify whether hind wings are present. In the diagnostic part the species was compared only with all wingless *Notaris*, from which it was distinguished by having “large, wide and concave body”. The species was believed to be “close to *N. bimaculatus* F.”, currently known as *Tournotaris bimaculata*. The type series consists of the male holotype and seven paratypes, all in ZIN, collected in mid-May 1901. The type locality is “China, basin of the Blue river (=the Yangtze), the Kundur-Tschu river, 13200”, which is in extreme north-western Sichuan (Fig. 9). We studied the holotype (Fig. 4A) and female paratype (Fig. 4B) of this species (without dissecting genitalia or attempting DNA extraction) and could not find reliable morphological characters to distinguish them from most of our freshly collected specimens.

An attempt to elucidate identity of *Notaroides brevirostris* was evenly inconclusive. The original description states that this wingless specimen is “rather similar with wingless *Notaris* species, but distinguishable by short, thick and straight rostrum with barely distinguishable scrobes and by concave “back” (this structure cannot be interpreted, perhaps “dorsal surface of rostrum”) above antennal attachments, as well as by smaller and convex eyes”. The specimen (Fig. 4C) was collected in early August 1900 and its type locality is “China, basin of the Blue river (=the Yangtze), lake Rkhombo-Mtsu valley, 13000”, which we interpreted as SE Qinghai (Fig. 9). Its short rostrum (Fig. 4C) is indeed unique to this taxon among all other flightless Asian *Notaris* (Figs 1–5). Its taxonomic status as a separate genus implies lack of close relationship to flightless *Notaris* from the same region, a hypothesis which appears as highly unlikely. Known from a single specimen, this obscure taxon will await its adequate study. It appears likely, however, that at least the generic name will be eventually synonymised under *Notaris* (a taxonomic action not implemented herein).



**FIGURE 8.** Ultrametric time tree obtained from Analysis 5 by using BEAST software to date evolutionary events of the *Notaris* + *Tournotaris* clade. Numbers at nodes and on the scale below are million years before present. Node bars represent 95% confidence interval of the age estimate. Alternating snowflake and sun symbols denote Pleistocene climatic fluctuations.

Both type localities are at about 4000 m in the region characterized by almost complete lack of deciduous forest. This, together with the opportunistic hand picking collecting style likely to be expected in Kozlov's expeditions (i.e. not using technically demanding litter sifting), suggest that these specimens were found in open alpine habitats similar to those of other herein reported *Notaris* from Southwest China (Fig. 10E–H) and Kazakhstan (Figs. 10I–L).

### Monophyly, distribution and time of origin of *Notaris* in Southwest China

In this study we advance a hypothesis that all flightless *Notaris* sampled from Southwest China form a clade (Fig. 6), as recovered in Analysis 2. In Analysis 1 a single sequence of volant Holarctic *N. aethiops* was nested within this clade, which was an odd result, considering conflicting geographical and biological scenario such relationships would imply. Analyses 3 and 4 were specifically performed to address this puzzling placement of *N. aethiops* inside Chinese *Notaris*. Removing this volant taxon from the analysis not only retained all Chinese *Notaris* as a clade, but also increased the ML bootstrap support almost three-fold (from 8% to 23%, Fig. 7). We interpret these results that (A.) flightless *Notaris* from Southwest China are monophyletic and (B.) placement of *N. aethiops* among them in A1 was a currently unexplainable artefact.

Recovery of monophyletic *Notaris* of Southwest China is the second significant “positive” results of the present study. This implies a number of hypothesis, among them that Southwest China is likely not the place where the more inclusive clade of *Notaris* + *Tournotaris* originated. Instead, Southwest China was more likely populated by *Notaris* in a single colonizing event coinciding with, if not linked to, the uplift of the Tibetan Plateau and adjacent landmass (Central Asia, Hengduan) and accompanying by drastic climate and biota changes (Favre *et al.* 2015) resulting from the Indian-Eurasian collision some 55 to 40 Ma.

The second implied hypothesis is that the origin of all analysed and presumably low dispersing wingless *Notaris* in Southwest China dates well before the onset of the Pleistocene climatic fluctuations (Fig. 8). This result corroborates the hypothesis that the Quaternary climatic changes do not represent the main driving force for the presently observed diversity in the region, although they are likely responsible for the middle- and high-altitude exile of *Notaris* in Southwest China. Similar conclusion has been reached for flightless high altitude *Niphadomimus* Zherikhin and *Trichalophus* LeConte weevils sympatric to *Notaris* in Southwest China (Grebennikov 2014a and 2015, respectively).

It should be noted that some other nearby localities seemingly suitable for *Notaris* (Mount Jizu, the Cang Shan Mountain Range and the Gaoiligong Shan Mountain Range; all in Yunnan; see sampling localities in Grebennikov & Smetana 2015) did not respond positively to diligent sampling. The latter fact is only suggestive and surely not

conclusive evidence of the lack of *Notaris*, since of course no amount of negative samples can conclusively demonstrate a lack of an organism. It should be also noted that in spite of seemingly similar conditions existing within an environment, specimen distribution pattern is often markedly uneven and so far unpredictable. The latter consideration is best exemplified by an observation that all 14 specimens of two not most closely related *Notaris* lineages known to inhabit deciduous forest litter on Mount Emei (Figs 1, 2B, 10A) were recorded in three separate samples taken within three days, while 24 other similarly sized samples taken in similarly identical Mount Emei habitats within three years failed to recover any.

Our results also imply that flightlessness observed on all *Notaris* in Southwest China is (1.) likely an evolutionary novelty synapomorphic to this clade and (2.) within the clade on *Notaris* + *Tournotaris* it evolved at least twice: in the most recent common ancestor of *Notaris* inhabiting Southwest China and in that of Central Asian *Notaris* represented in our analysis by a single species from Kazakhstan (more on this radiation is below).

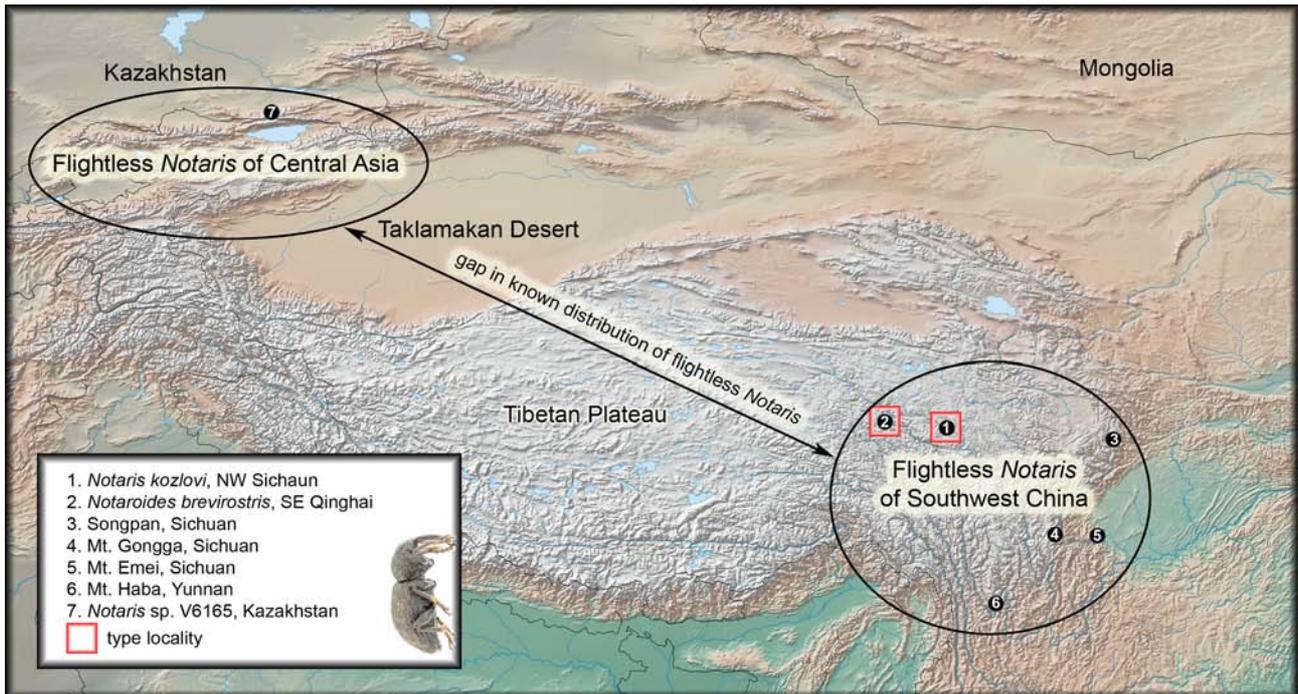
### **Species concept and rates of CO1 nucleotide substitutions of *Notaris* in Southwest China**

This paper demonstrates difficulties of using Linnaean taxonomy for inadequately known organisms, further obscured by existing of historical names. During gradual accumulation of *Notaris* specimens from Southwest China we had a notion that each locality supports at least one “species”, similarly to taxonomic commonly patterns adopted for a number of other mountainous radiations of low-dispersing organisms, beetles in particular (Belousov 1998). This hypothesis started, however, to erode as we became unable to distinguish specimens from different localities not only externally, but also using their genital characters. Results of DNA barcoding were equally inconclusive, since each of four sampled localities supporting *Notaris* populations in Southwest China (Mount Emei, Songpan, Mount Gongga and Mount Haba, Fig. 9) was a home to two genetically notably distinct lineages (Figs 7, 8), which in two cases (Songpan and Mount Emei) did not form a clade (Fig. 7). In view of these uncertainties on one hand and great external similarity between newly sampled specimens and those of narrowly parapatric *N. kozlovi* on another, we decided to unite all these specimens under a single broadly distributed, likely monophyletic and relatively polymorphic species, for which a taxonomic name (*kozlovi*) was readily available. Any alternative approach, such as either forcing new names on these inadequately known beetles or leaving them unassigned to a Linnaean species seem markedly less advantageous.

An interesting spin off from our results is an observation that *N. kozlovi*, as this species is presently re-defined, originated about 14.4 Ma. This clade is, therefore, about twice greater than the strongly supported clade of three Palaeartic volant species: *N. acridulus*, *N. scirpi* and *N. puncticollis* (LeConte) (Fig. 8) dated to only about 7.3 Ma. Even more remarkable, 5.1 Ma separation between *N. acridulus* and *N. scirpi*, two well-established “good” species, it supposed to take place almost simultaneously with that of two distinct lineages of mitochondrial DNA sequenced from morphologically undistinguishable specimens of *N. kozlovi* living in the alpine zone of Mount Haba and often found under the same stone. One of possible reasons for this observed discrepancy might be that linear molecular clock hypothesis, as implemented in our Analysis 5 to date *Notaris* evolutionary events (Fig. 8) is not the most applicable for a clade which includes both flightless and volant lineages. More specifically, DNA evolution might likely go at an accelerated rate in flightless organisms such as *N. kozlovi*, as compared to other flight-capable *Notaris* (Mitterboeck & Adamowicz 2013; Welch & Bromham 2005). This supposition is in an agreement with the rates reported for the 3-end CO1 fragment (=DNA barcoding) of flightless *Trigonopterus* Fauvel, which might be as high as 0.0342 subs/s/Ma/l or even 0.0865 subs/s/Myr/l (Tänzler *et al.* 2016), which is 2–5 times greater than the rate used in our Analysis 5. Too little, however, is currently known on the rates of DNA evolution to be dogmatic in this respect (Bromham 2008).

### **Diversity and distribution of Central Asian *Notaris***

The assessment of the presently known diversity of *Notaris* strongly advocates that at least in two areas the genus is represented by numerous allopatric flightless high altitude and non-riparian lineages. One of these regions might be the southeastern fringes of the Tibetan Plateau (Fig. 9, the Hengduan Mountains and the adjacent areas of the Tibetan Plateau). A second and seemingly separate region known to support higher than usual diversity of *Notaris*



**FIGURE 9.** Distribution of flightless *Notaris* in Asia. The gap between two circled areas is likely a sampling artefact.

is the highlands of the Central Asia, mainly in the Tian Shan system of mountain ranges (Fig. 9). Caldara (2011) reports six species for this region: *N. acridulus* Linnaeus, *N. discreta* Faust, *N. imprudens* Faust, *N. nivalis* Faust, *N. oberti* s. str. Faust and *N. scirpi* Fabricius, all of which have been previously cited by Bajtenov (1974) for the territory of Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan and Turkmenistan. Bajtenov, who lived in Almaty and worked specifically on the Central Asian weevils, did not cite *Notaris* specimens that he had actually studied. After his death his collection was inherited by relatives and most likely lost completely, thus the exact identity of most of his data cannot be presently verified. With the exception of the first and the last species, which are widely distributed winged ones, all the others were described by Faust in 1882 and 1885 from specimens collected mainly by Russian governmental officials during the rapid imperial invasion of the region (Pierce 1960). They, most likely, represent high altitudinal flightless Central Asian endemics not dissimilar in many respects to those presently discovered in Southwest China. Identification of these four Faust's species from Central Asia and those from the nearby areas of Siberia, Mongolia and China, even though they are repeatedly cited for the region for nearly 130 years, requires consulting the type specimens, which has presumably never been done. It is possible that the *Notaris* sp. V6165 (Figs. 9, 12, 13, 14) collected just 10 km from the municipal boundaries of Kazakhstan's former capital Almaty (former Verniy, the type locality for many of Faust's species) already existed under Faust's name that, however, cannot be verified without consulting the historical specimens. On the whole, this situation with a multitude of historical *Notaris* names represents a case of "taxonomic impediment", when the problem of species discovery is further and most significantly obstructed by the necessity to clarify and use inadequately known historical names.

Existence of two separate mountain regions with a high *Notaris* diversity in Asia might be an artefact of inadequate sampling and knowledge. The genus might be much more widely distributed in northwestern China (and perhaps in Central Asia) than presently known. The geographical gap seemingly existing between both hypothesised regions (i.e. between the Tian Shan mountain ranges and the Hengduan Mountains, Fig. 9) might be eventually bridged by new *Notaris* finds. Independent loss of hind wings coupled with acquiring biological association with high altitude non-riparian habitats (as implied by the results of the phylogenetic analysis; Fig. 7) might also prove later to be a sampling or analytical artefact. It appears likely that all wingless and high altitude *Notaris* in Asia might form a clade, even if such a hypothesis is presently refuted by the analysis. Single loss of wings agrees with the phylogeographical and biological evolutionary scenarios elucidated below and might be eventually demonstrated as the most parsimonious phylogenetic hypothesis.



**FIGURE 10.** Three novel habitat types of *Notaris* weevils; red rectangles indicate the exact spot where specimens were detected. A: leaf litter in the mountainous deciduous forest on Mount Emei, Sichuan, the collecting spot of V1207; B: leaf litter in the mountainous *Rhododendron*-dominated shrub on Mount Gongga, Sichuan, the collecting spot of the specimen V2375, note the glacial moraine in the foreground; C–D: leaf litter in the mountainous *Rhododendron*-dominated shrub in vicinities of Songpan, Sichuan, the landscape (C) and the collecting spot of V5376 and V5377 (D); E–H: rocks in the alpine zone on Mount Haba, Yunnan (F–H: together with a weevil molytine larva of the genus *Niphadonyx* Schenkling); I–L: rocks in the alpine zone of Trans-Ili Alatau (=Zailiysky Mt. Range), Kazakhstan, habitat of *Notaris* sp., including V6165.

## Evolution of habitat association in *Notaris* and *Tournotaris*

While nothing is known on the host plants for the newly discovered flightless Chinese *Notaris*, they are expected to develop on monocots, the same way as the majority of other Eirrhinini with known host plants. Egorov *et al.* (1996: 434) indicated that larvae of both *Notaris* and *Tournotaris* (under the names *Eirrhinus* and *Notaris*, respectively) develop inside riparian monocots. Westra *et al.* (1981) made an exceptionally well documented report on the *T. bimaculata* lifecycle on quackgrass (*Elymus repens* (L.) Gould), while extensive biological review of European species might be found in Gültekin (2013).

Most Eirrhinini species are thought to favour moist habitats (Thompson 2006). This noted moisture and even water association, along with having grasses as their host plants, might have predisposed some Eirrhinini to become rice pests when dispersing human populations had offered such an opportunity (Lyal 1990). Caldara & O'Brien (1995) designated the vast majority of the Asian Eirrhinini (including all *Notaris* under the name *Eirrhinus*) as “semiaquatic and aquatic”. This generalization is not corroborated by observations of the habitats for the newly described Chinese species, which all have been found in not particularly wet places. Besides, at least some of the Central Asian *Notaris* species (such as *N. imprudens* Faust) were described without adequate knowledge of their habitat associations and because they were not knowingly re-sampled afterwards, nothing is actually known of their biology.

The present study establishes a few novel *Notaris* biological traits and suggests their possible evolutionary interpretation. Populations of *Notaris* studied for the present report firmly ties in by three parameters: (1) mid- and high altitude habitation 2440–4195m; (2) complete loss of hind wings likely synonymous with low dispersal capacity and (3) no specific association with moist habitats. Some of these observations might perhaps be partly traced to the original descriptions of other and similarly altitudinal species (see Faust 1885; Korotyaev 1979; Legalov 1997). None of these authors, however, collected actual specimens of their new respective species and were, therefore, unaware of their specific biological associations. For this reason, the newly reported facts strongly contrast with the repeated observations on the riparian lifestyle common among the winged lowland *Notaris* and other species attributed to Eirrhinini.

A second novel observation is that at least flightless *Notaris* can be collected in Southwest China by sifting forest litter in the alpine *Rhododendron* L. shrubs (Songpan, Figs 10C, D; Mount Gongga, Fig. 10B) and the broad leaf forest (Mount Emei, Fig. 10A) habitats. As far as it is possible to ascertain, never before have *Notaris* species been associated with this type of habitat.

It seems plausible to suggest that alpine- and forest-zone litter *Notaris* associations can be phylogenetically interpreted as modifications taking place after the lineage has already changed its originally open wet lowland habitation to the open and not-so-wet alpine habitats. In other words, the direction of evolutionary changes was from the original wet open lowland habitats to drier open altitude habitats, followed by the invasion into the alpine litter and then into the forest litter habitats; the latter two steps occurred either in this sequence, or in parallel. The apparent difficulty with this hypothesis (that the low altitude species bypass the forest zone when becoming the higher altitude alpine species) can be relieved by an observation that Central Asia was historically short of deciduous forests, their scarcity owing to the relatively low rainfall since at least mid-Miocene (Favre *et al.* 2015), or even perhaps much earlier (Harris 2006). Besides, it is unlikely that the change from the low altitude to the higher one was achieved by the species extending their distribution and actually climbing up through the forests along their way to the alpine zone. Instead it seems plausible that the ancestor of the Yunnan and Sichuan *Notaris* might have been caught in the tectonic uplift of the Tibetan plateau which elevated and changed beetles' habitat to its present high altitude open areas without ever becoming forested or glaciated. As for the two true forest *Notaris* lineages both inhabiting Mount Emei, the subsequent change in their biological associations from open high altitude habitation to the deciduous forest zone could have been gradual though *Rhododendron* shrub litter, the habitat presently inhabited by other in-group *Notaris* (Fig. 7). The final push of *Notaris* on Mount Emei into the deciduous forest litter might have been facilitated by this mount entirely losing its once open summit habitats and becoming fully forested up to the top. The latter change, in turn, could have been facilitated by the erosion which gradually lowered the mount and was coupled with altitudinal fluctuation of the upper forest line. The latter could have been positively correlated with the amount of precipitation linked to the Pleistocene climate cycles and having one of its maxima during the current Holocene epoch. If true, this will suggest that in the situation when both the alpine zone and the deciduous forest are present, the *Notaris* species in Southwest China are more likely to be

found in the former rather than in the latter, as exemplified by *Notaris* on Mount Haba. This change of habitats scenario should be treated as an untested and preliminary assumption, since the underlying phylogenetic hypothesis (Fig. 7) is far from being fully and consistently resolved. Additional difficulty is offered by the presently unexplainable observation of *Notaris* on Mount Gongga inhabiting the deciduous forest and *Rhododendron* shrub litter (Fig. 7) in a situation when the adjacent and likely stable open alpine landscapes failed to produce a single specimen of this species during a two day search. Overall, however, the proposed evolutionary scenario of biological changes presently forms the best fit to the available data and should be considered as the most likely, until refuted.

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