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Cryptic species of the *Myrmica tibetana* complex (Hymenoptera: Formicidae) revealed by integrative taxonomy

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Abstract

Three closely related species, *Myrmica tibetana* Mayr, 1889, *M. bactriana* Ruzsky, 1915 and *M. gebaueri* sp.n., are identified. They are restricted to the Tibetan Plateau and proposed to form the *M. tibetana* species complex. *Myrmica tibetana* and *M. gebaueri* sp.n. are truly cryptic: They showed considerable interspecific overlap in all of the tested 18 shape, pilosity, and sculpture characters and were not safely separable by simple visual inspection by a trained expert. However, all three entities are clearly demonstrated by Nest Centroid (NC) clustering of morphological data which agreed by 100% with the genetic classification based on 11 microsatellite markers. The clusters shown by hierarchical NC-Ward clustering and the partitioning algorithms NC-part.hclust and NC-part.kmeans were coincident in all of the 62 nest samples. A stepwise linear discrimiant analysis reducing the set to nine characters achieved a classification error of 0% in 178 investigated worker individuals. All three entities are partially sympatric, and the absence of phenotypically mixed nest samples rejects the hypothesis that they could represent an intraspecific polymorphism. The coincident classification of all three exploratory data analyses of morphology and nuDNA revealed a paraphyly of mtDNA between *M. bactriana* and *M. gebaueri* sp.n. adding another example to the multiple evidence on failures of mtDNA barcoding in biodiversity research. Yet, mtDNA data appeared adequate for rough assessment of divergence times. According to this, the separation of the *M. tibetana* complex from other members of the *M. rubra* group is estimated to have occurred approximately 7.5 Ma Before Present (BP), and the radiation within the *M. tibetana* complex started > 5 Ma BP. A taxonomic description and a differential diagnosis of *M. gebaueri* sp.n. are presented. *Myrmica bactriana* Ruzsky, 1915 is shown as a senior synonym of *M. furva* Ruzsky, 1915 and *M. ruzskyana* Radchenko & Elmes, 2010. Synonymies of either member of the *M. tibetana* complex with the following Central and Middle Asian species are excluded: *M. smythiesii* Forel, 1902, *M. fortior* Forel, 1904, *M. wittmeri* Radchenko & Elmes, 1999, and *M. tenuispina* Ruzsky, 1905.

Key words: Nest centroid clustering, species delimitation, numeric morphology-based alpha-taxonomy, phylogenetic age, Tibetan fauna, microsatellite primer sequence, new species, new synonymies.

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Introduction

There are disparate conceptions of what "cryptic species" might constitute. True crypsis is given when heterospecificity has been clearly demonstrated by some kind of an exploratory data analysis and if an experienced expert is not able to use these samples with pre-established determination to build up in her/his brain pathways for reliable subjective-sensory species recognition. This permanent, true crypsis differs from temporary or historic crypsis. The meaning of the latter can be elucidated by an example from ornithology: The Common Tern *Sterna hirundo* and the Arctic Tern *Sterna paradisaea* were considered inseparable by field observers in the 1950s. Today, however, hundreds of amateur ornithologists in Europe are able to reliably identify these two species in the field because the knowledge on useful characters and individual training have enormously developed. In order to distinguish from temporal crypsis, Seifert (2009) defined true crypsis as follows: "Cryptic species are two or more species which are not separable by primary visual or acoustic perception of an expert. This reflects the immediate sense of the word and restricts such species to truly cryptic cases – i.e., to species not safely separable by training of innate pathways of the human cognitive system. Rather, their reliable identification requires the application of elaborate methods such as numeric recording and analysis of phenotypic characters, DNA analysis, biochemistry or analysis of sound spectrograms …"

Within the context of the Chinese-German research project Pasture Degradation Monitoring System (PaDeMoS) three of the authors conducted field work in grassland ecosystems on the Tibetan Plateau in 2011 and 2012. The ant fauna of this huge, climatically extreme area is poorly studied. This also applies to the genus *Myrmica* Latreille, 1804 of which we collected ten species. Among these was *Myrmica tibetana* Mayr, 1889, originally described from material of the famous 1884 Przewalski expedition near Lake Kuku Nor (Qinghai Lake) in NE Tibet and the closely related *M. bactriana* Ruzsky, 1915, collected during the Kozlov expedition in 1900 / 1901. To our surprise, we discovered a third sympatric species very closely related to *M. tibetana*. Two of these are truly cryptic following the definition given above: They showed considerable interspecific overlap in any of the tested 18 shape, pilosity or sculpture characters and were not safely separable by simple visual inspection of a trained expert. Such non-transparent situations can be solved by application of advanced exploratory data analyses. In eusocial organisms, Nest Centroid clustering (NC clustering) is the method of choice. NC clustering has been introduced by Seifert & al. (2014) and is promising to be a powerful tool for recognition of taxonomic and zoogeographic patterns for any cohesive organism or social system providing repeats of definitely conspecific elements. A dozen of follow-up applications of NC clustering using data of Numeric Morphology-Based Alpha-Taxonomy (NUMOBAT; e.g., Seifert 2013, Csösz & al. 2014, Seifert & al. 2014, Csösz & al. 2015, Seifert & al. 2017a, b) and another one using data of cuticular hydrocarbon chemistry of ants (Guillem & al. 2014) have been published since then. Speed and objectivity of hypothesis formation has recently been improved by introducing Partitioning Algorithms based on Recursive Thresholding (PART) into the NC-clustering protocol (Csösz & Fisher 2015). Here, we present a convincing demonstration of truly cryptic and sympatric *Myrmica* species by NC clustering of NUMOBAT data and support this finding by a classification based on nuclear DNA.

Material

NUMOBAT data were recorded in a total of 62 nest samples and 178 worker individuals collected on the Tibetan Plateau. In the individual species treatments, material examined is listed in following sequence and format: site, date in the format yyyy.mm.dd, field sample number "field No" which is found on the mounted specimens (the sample number of microsatellite DNA analysis "µsat No") (GenBank accession number) [latitude in decimal format, longitude in decimal format, metres above sea level]. The accuracy of coordinates is proportional to the number of decimal points and "xx" in the sampling date sequence mean missing data. In some samples without any direct or derived information on date, the collector is given to allow an approximate conclusion on the time period of collection. Field sample numbers are missing in some historic samples.

The acronyms of depositories are as follows:

NHM Wien – Naturhistorisches Museum Wien, Austria
MHN Genève – Muséum d'histoire naturelle de Genève, Switzerland
SMN Görlitz – Senckenberg Museum für Naturkunde Görlitz, Germany
ZM Berlin – Zoologisches Museum am Museum für Naturkunde Berlin, Germany
ZM Moskow – Zoological Museum of the Lomonossov University Moskow, Russia
ZM St. Petersburg – Zoological Museum St. Petersburg, Russia

Myrmica tibetana Mayr, 1889

A total of 12 nest samples with 45 workers were investigated by linear morphometrics.

Ganzu: Aze Station, riverbank, 2012.07.10, field No 032 (= µsat No M29) (GenBank MG603025), field No 033 (= µsat No M30) (GenBank MG603026) [33.655° N, 101.831° E, 3478 m]; Luqu 8.4 km SW, 2011.08.19, field No 074 (= µsat No M12) (GenBank MG603020) [34.541° N, 102.416° E, 3297 m]. **Qinghai:** Hainan-10 km NE, 2011.09.14, field No 107a (GenBank MG603022) [36.354° N, 100.708° E, 3088 m]; Lake Kuku Nor, 2011.07.28, sample No 027 (= µsat No M07) (GenBank MG603019) [37.144° N, 99.752° E, 3206 m]; Beishan National Park, 1996.05.25 [36.95° N, 102.48° E, 2400 m]; Heka, 1990.07.14 [35.78° N, 99.88° E, 3560 m]; S Kuku Nor Mountains, type *M. tibetana* 1884.04. [36.5° N, 98.7° E, 3700 m]; Xia Zhangshan S, 2012.07.05, field No 023a (= µsat No M25) (GenBank MG603024) [35.592° N, 102.711° E, 3032 m]; Xinghai 2 km NNW, 2011.07.17, field No 56 [35.605° N, 99.978° E, 3314 m]; Xinghai, Stipa, 2012.07.01, field No 010 (GenBank MG603023), field No 011 (= µsat No M23) (GenBank MG603021) [35.605° N, 99.981° E, 3305 m].

Myrmica bactriana Ruzsky, 1915

A total of 20 nest samples with 52 workers were investigated:

Ganzu: Aze Station, rock, 2011.08.14, field No 056b [33.681° N, 101.874° E, 3605 m]; Aze Station, rock, 2011.08.14, field No 058 (= µsat No M11) (GenBank MG603009) [33.681° N, 101.873° E, 3608 m]; Aze Station 3.3 km NNE, 2011.08.12, field No 172, field No 173 (= µsat No M16) (GenBank MG603011), field No 174 (= µsat No M17) (GenBank MG603012), field No 175 [33.702° N, 101.887° E, 3630 m]; Aze Station, above AzAII, 2012.07.09, field No 027, field No 028 (= µsat No M27) (GenBank MG603017), field No 029 (= µsat No M28) (GenBank MG603018) [33.676° N, 101.854° E, 3599 m]; Aze Station, near AzAII, 2012.07.08, field No 025a (= µsat No M26) (GenBank MG603016) [33.667° N, 101.854° E, 3551 m]; Luqu 8.4 km SW, 2011.08.19, field No 078 (=µsat No M13) (GenBank MG603010) [34.541° N, 102.416° E, 3295 m]; Luqu 8.4 km SW, plot LqA, 2011.08.17, field No 177, field No 181 (= µsat No M18) (GenBank MG603013), field No 183 (= µsat No M19) (GenBank MG603014), field No 185a (GenBank MG603015) [34.541° N, 102.418° E, 3296 m]; **Qinghai:** Aba County, wetland, 2011.08.20, field No 081 [33.455° N, 101.840° E, 3520 m]; Sanzin Gömpa Monastery, creek, 2011.07.21, field No 021 (= µsat No M06) (GenBank MG603008) [35.501° N, 99.824° E, 3517 m]; Sanzin Gömpa Monastery -1.7 km E, field No SaA-BPs, 2011.07.18 [35.500° N, 99.817° E, 3588 m]; Sanzin Gömpa Monastery-2.2 km E, field No SaZ-Blg, 2011.07.20 [35.504° N, 99.823° E, 3628 m]; **Sichuan:** river Dza-Chyu, 1901.05, type *M. furva* [32.930° N, 98.770° E, 4080 m].

Myrmica gebaueri sp.n.

A total of 30 nest samples with 81 workers were investigated:

Ganzu: Tjanzhu-0.5 km SSE, 2011.08.03, field No TzA Blg [37.192° N, 102.788° E, 2901 m]; Tjanzhu-0.8 km SSW, 2011.08.04, field No 041 (= µsat No M08) (GenBank MG603000) [37.189° N, 102.783° E, 2963 m]; Tjanzhu-0.8 km

SSW, 2011.08.04, field No TzZ Blg, field No TzZ-R, field No 044 (= µsat No M09) (type series of *M. gebaueri* sp.n. GenBank MG603001) [37.189° N, 102.783° E, 2963 m]; Tjanzhu, 2011.08.05, field No 049 (= µsat No M10) (GenBank MG603002) [37.182° N, 102.776° E, 3095 m]; Tjanzhu, Gipfel, 2011.08.04, field No 84 [37.177° N, 102.773° E, 3146 m]; Xicheng, 2011.08.01, field No 037b [38.038° N, 101.593° E, 3167 m]; **Qinghai:** Heimahe - 4.1 km SSE, 2011.07.26, field No KoA2 - B1 (= µsat No M31) (GenBank MG603006), field No KoA2 - Blg (GenBank MG603007) [36.696° N, 99.802° E, 3294 m]; Heimahe - 4.4 km SSE, 2011.07.24, field No 64, field No KoZ - BPs, field No KoZ-pseu [36.694° N, 99.804° E, 3288 m]; Heimahe - 4.4 km SSE, 2011.07.26, field No KoZ2-Blg, field No KoZ2-R1, field No KoZ2-R2, field No KoZ2-Re [36.691° N, 99.797° E, 3294 m]; Sanzin Gömpa Monastery 6 km NE, 2011.07.18, field No 016 (GenBank MG602998), field No 017 (= µsat No M05) (GenBank MG602999) [35.542° N, 99.840° E, 3435 m]; Sanzin Gömpa Monastery 6 km NE, 2012.07.03, field No 020, field No 021 (= µsat No M24) (GenBank MG603005) [35.541° N, 99.849° E, 3409 m]; Gonghe, 1992.05.08 [36.300° N, 100.683° E, 3400 m]; Xinghai-13 km WSW, 2011.07.19, field No 61, field No 62 [35.542° N, 99.840° E, 3428 m]; Xinghai, Stipa, 2012.07.01, field No 008b [35.610° N, 99.977° E, 3332 m]; Xinghai, Stipa, 2012.07.01, field No 009 (= µsat No M21) (GenBank MG603004) [35.607° N, 99.982° E, 3311 m]; Xinghai, grassland, 2011.07.17, field No 013a (= µsat No M01) (GenBank MG602995), field No 014 (= µsat No M02) (GenBank MG602996), field No 015 (= µsat No M03) (GenBank MG602997) [35.617° N, 99.975° E, 3374 m]; Xinghai 3 km NNW, plot XiZ R, 2011.07.16, field No 158a [35.612° N, 99.968° E, 3349 m].

Detailed information on type material

***Myrmica tibetana* Mayr, 1889**

Mayr (1889) gave the following collecting data in his original description: "April 1884, Jumel-Kuku-Gebirge; Mai-Juni 1884 Tibet septentr." The term "Jumel Kuku Gebirge" is undoubtedly a reading error of the original Cyrillic label. This original label was discarded by Mayr and should have read probably as "Южные Куку Горы" = "Southern Kuku Mountains". If handwritten as a script, "Южные" is easily misinterpreted by a person not familiar with Russian language. Reading the travelling report of Przewalski (1954), we found that the Southern Kuku (Nor) Mountains were reached in April 1884 and we assume as most probable collecting site a place near the pass road – approximately at 36.5° N, 99.7° E and 3700 m.

We have investigated the lectotype worker labeled "Tibet" [handwriting of H. Stitz], "Myrmica tibetana Mayr" [handwriting of H. Stitz], "Forel ded. 1922", "Zool. Mus. Berlin", "Paratypus" [label probably attached by Stitz], "Lectotype *Myrmica tibetana* Mayr, 1889 [published by Radchenko & Elmes (2010), des. Seifert 2014]"; stored in ZM Berlin. Note: Radchenko & Elmes (2010) published a specimen from ZM Berlin museum with the above labelling as lectotype but did not physically designate it and they also gave no morphological data to identify it unambiguously. However, as this type is the only specimen of *M. tibetana* stored in the Berlin collection, we are rather sure to have labeled the right specimen. We further investigated a big series of 13 paralectotype workers, stored in NHM Wien, labeled "Tibet Coll. G. Mayr", "tibetana G.Mayr, Type". One of these specimens shows a label "Lectotype Myrmica tibetana MAYR A.F. -1978" which is invalid as this physical lectotype designation by André Francoeur has not been published.

***Myrmica gebaueri* sp.n.**

Holotype labeled "CHI: 37.1852° N, 102.7844° E Tianshu station-1.2 S, 2939 m moist pasture, under stone R.Schultz 2011.08.04-044" and "Holotype *Myrmica gebaueri* Seifert et al. 2018"; two worker paratypes on a different pin, 21 worker and 14 male paratypes stored in ethanol – all from the same nest sample and with equal collecting data label as the holotype; all material stored in Senckenberg Museum für Naturkunde Görlitz. Three worker paratypes with the same labelling in MHN Genève.

***Myrmica tibetana* var. *furva* Ruzsky, 1915**

We investigated three supposed paralectotype workers from ZM St. Petersburg, labeled "r. Dza-Chyu, Kam', Golubaya, 12 - 12500', Kozlov, nach. V.01" [in Cyrillic] and "Myrmica tibetana Mayr M. Ruzski det." Radchenko & Elmes (2010) published a lectotype with identical locality label. This lectotype was not found in the St. Petersburg museum. Perhaps it was not physically labeled by Radchenko & Elmes. In each of the three paralectotypes some characters could not be recorded due to damage – in one the whole head was missing. However, we constructed complete data sets for two specimens using relational calculations. River Dza-Chyu is a tributary of the upper Yangtse (= Golubaya in the Russian naming of the Kozlov expedition). According to the travelling report of the expedition (Kozlov 1906), the putative collecting site was reached 11 May 1901 and should be situated approximately at 32.928° N, 98.770° E and ca. 4080 m if the lowest point in that region is chosen. The 12 - 12500 feet given on the label are equal to 3750 m. This means no contradiction because altitudinal estimates in Kozlov's time were rather inaccurate.

***Myrmica smythiesii* var. *bactriana* Ruzsky, 1915**

Radchenko & Elmes (2010) published a lectotype worker stored in ZM St. Petersburg and cite its label as "okr. ur. Darindo, Kam, verkh. Goluboj, Kozlov, 1/3.VIII.00" [in Cyrillic]. The term "1/3.VIII.00" stands probably for the first decade of August (I. Kabak, pers. comm.). This site is situated at the upper course of Yangtse at 33.054° N, 96.903° E and 3850 m. No type specimens could be discovered in the collection St. Petersburg during a search by D. Dubovikov in 2013 but the identity of this taxon and of *M. ruskyana* Radchenko & Elmes, 2010 can be concluded with low risk of error from Radchenko & Elmes drawings of the lectotypes and the geographic data (see section Results and Discussion).

***Myrmica ruzskyana* Radchenko & Elmes, 2010**

This is a replacement name for the primary homonym *Myrmica smythiesii* var. *exigua* Ruzsky, 1915. Radchenko & Elmes (2010) published a lectotype labeled "rechka Bachyu, 12.000', Kam, bass. Goluboj r., Kozlov, 2/3. VIII. 00" [in Cyrillic]. "2/3. VIII". means probably the second decade of August (I. Kabak, pers. comm.). Though the label shows another locality name, the travelling report of Kozlov does not allow separating this site geographically from the lectotype locality of *M. bactriana*. According to Kozlov's map, he had been in Darindo (locality of

M. bactriana) on 8 August and in Ba-Tshu River on 9 - 20 August 1900. The linear distance between Darindo and the mouth of Ba-Tshu River is approximately 11 km and that between Darindo and the next station – the confluence of the Ba-Tshu and Dza-Tshu rivers, reached on 21 August – is about 27 km (I. Kabak, pers. comm.). Thus the collecting points are between 11 km and 27 km apart and both in the Yangtse basin close to the present town of Yushu. Type material was not available from ZM St. Petersburg and ZM Moscow.

***Myrmica tenuispina* Ruzsky, 1905**

The combination *Myrmica laevinodis* var. *tenuispina* Ruzsky, 1915 is the first available use of *Myrmica rubra laevinodis tenuispina* Forel, 1904 and the types are those designated by Forel. Four syntype workers from MHN Genève were investigated, labeled "*M.rubra Linné r. laevinodis Nyl. v. tenuispina For type Buchara*" [Forel's handwriting] and a printed label in Cyrillic letters "Tabi dara-Zagyr-desht. v. Bukhara Kaznakov 17 VI. 97". These specimens belong to the lectotype sample because Radchenko & Elmes (2010) published a lectotype worker in the ZM Moscow with the labelling "Tabi-Dara Zagyrdesht V.Buchara, 17. VI. 97, Kaznakov" [in Cyrillic].

Methods

Phenotypic investigation

The optical equipment used, the character recording methods and estimation of measuring errors are given in Seifert (2011). Precise definitions of the following phenotypical characters are given in Seifert & al. (2014). Briefly explained these are: cephalic length CL, cephalic width CW, head size CS (= arithmetic mean of CL and CW), eye size EYE (= arithmetic mean of large and small diameter of the elliptic eye), scape length SL, maximum frontal lobe width FL, minimum frontal carina distance FR, petiole width PEW, postpetiole width PPW, petiole height PEH, petiole length PEL, maximum length of postpetiolar setae PPHL, spine length SP, metapleural lobe height MetL, height of subspinal propodeal excavation MetSp and postocular distance PoOc. We introduced the following new characters in the investigation system of the *Myrmica tibetana* complex and present their precise description:

SPBA – the smallest distance of the lateral margins of propodeal spines at their base. This should be measured in dorsofrontal view, since the wider parts of the ventral propodeum do not interfere with the measurement in this position. If the lateral margins of spines diverge continuously from the tip to the base, a smallest distance at base is not defined. In this case, SPBA is measured at the level of the bottom of the interspinal meniscus.

SPTI – the distance of propodeal spine tips in dorsal view; if the tips are rounded or thick, the centres of spine tips are taken as reference points.

Removal of allometric variance. Removal of allometric variance (RAV) was performed with the procedure described by Seifert (2008). RAV is calculated here for the assumption of all individuals having an identical cephalic size of CS = 950 µm. The parameters of RAV functions were calculated as the arithmetic mean of the species-specific functions of *Myrmica bactriana*, *M. gebaueri* sp.n. and *M. tibetana*. It can be seen from the functions below that allometries of shape are weak in the small and weakly size-variable workers of the *M. tibetana* complex. The RAV functions were as follows:

$CL / CW_{950} = CL / CW / (-0.0515 * CS + 1.1754) * 1.1265$
$SL / CS_{950} = SL / CS / (-0.0480 * CS + 0.8321) * 0.7865$
$EYE / CS_{950} = EYE / CS / (0.0528 * CS + 0.1455) * 0.1966$
$FL / CS_{950} = FL / CS / (-0.0055 * CS + 0.4786) * 0.4734$
$FR / CS_{950} = FR / CS / (-0.0459 * CS + 0.4421) * 0.3985$
$PEW / CS_{950} = PEW / CS / (-0.0605 * CS + 0.3063) * 0.2488$
$PPW / CS_{950} = PPW / CS / (-0.0630 * CS + 0.4377) * 0.3778$
$PEH / CS_{950} = PEH / CS / (-0.0293 * CS + 0.3464) * 0.3186$
$PEL / CS_{950} = PEL / CS / (-0.0862 * CS + 0.5346) * 0.4527$
$PPHL / CS_{950} = PPHL / CS / (-0.0408 * CS + 0.2370) * 0.1982$
$SPBA / CS_{950} = SPBA / CS / (-0.0350 * CS + 0.3182) * 0.2849$
$SPTI / CS_{950} = SPTI / CS / (-0.1597 * CS + 0.4595) * 0.3077$
$SP / CS_{950} = SP / CS / (0.0122 * CS + 0.1610) * 0.1726$
$MetL / CS_{950} = MetL / CS / (0.0001 * CS + 0.2228) * 0.2229$
$MetSp / CS_{950} = MetSp / CS / (-0.0006 * CS + 0.1980) * 0.1975$
$PoOc / CL_{950} = PoOc / CL / (-0.0703 * CS + 0.4774) * 0.4106$
$FL / FR_{950} = FL / FR / (0.1214 * CS + 1.0730) * 1.1882$

Analysis of phenotypic data. Exploratory data analysis was run using three different methods of NC-clustering (Seifert & al. 2014). These were hierarchical NC-Ward clustering and two partitioning algorithms based on recursive thresholding: part.hclust and part.k means (for details see Csösz & Fisher 2015). Checking for misclassified samples was done following the rationale described in Seifert & al. (2014). All linear discriminant analyses were run with the SPSS 16.0 software package.

Genetic investigation

The reduced number of 32 / 26 nest samples for which mtDNA / microsatellite data were evaluated compared to 62 nest samples available for morphological investigation is explained by the fact that DNA was extractable in only a part of these samples currently housed in museum collections. We did not made any attempt to extract DNA from mounted specimens because of the resulting physical damage. In the type specimens of *Myrmica tibetana* and *M. furva* such destructive investigations were even forbidden by the curators.

Analysis of mtDNA. From each genetically evaluable nest sample one worker was chosen for genetic analyses. The complete individual was shred with a mixer mill (Retsch MM 400, Haan, Germany) and genomic DNA was extracted using Qiagen DNeasy blood & tissue kit (Qiagen, Hilden, Germany).

An approximately 2.500 bp fragment of mtDNA was amplified in two segments. The first segment including *ND6* (89 AA), *cytb* and tRNASer was amplified using the primers cytb FeF (Liautard & Keller 2001) + tRS (Jermiin & Crozier 1994). The second segment including *ND1* (269 AA) was amplified using the primers ND6_ND1bF + ND6_ND1cR (Holzer & al. 2009). The second primer pair failed for all *Myrmica tibetana* specimens. Yet, since this taxon was already well separable from both *M. gebaueri* sp.n. and *M. bactriana*, no effort was undertaken to find new primer pairs. The short tRNASer sequence showed little variation and was ignored in this analysis.

Each segment was sequenced on both strands using the same primers. The segments overlapped at ca. 400 bp, and this sequence section was carefully checked for congruence between the two segments to potentially detect pseudogenes. Half of the individuals were additionally amplified with a third primer pair, CB11059 (Goropashnaya & al. 2004) +

Tab. 1: Microsatellite loci developed for *Myrmica tibetana,* which were cross-amplified for *M. bactriana* and *M. gebaueri*. Superscript letters at locus names indicate the combination of loci in multiplex PCR reactions. Annealing temperatures multiplex reactions 1 - 4: 1 at 63 °C; 2 and 3 at 61 °C; 4 at 59 °C.

Locus	Repeat motif	Size range (bp)	Primer sequence
B04[1]	$(TTGG)_{13}$	231 - 252	B04-fwd: 5‘-5AGATCGAGCCGGAGAATCG-3‘ B04-rev: 5‘-TACCTTCTCGTCGCCCAAC-3‘
B09[2]	$(GAAC)_{14}$	247 - 267	B09-fwd: 5‘-CCATTAGCGCGTCCAACAG-3‘ B09-rev: 5‘-ACCGAGGACTTCGTTAGGC-3‘
B10[3]	$(TCGT)_{18}$	263 - 282	B10-fwd: 5‘-GCGACAAGGAGAGCAAGTC-3‘ B10-rev: 5‘-AGAGCAGCATGAGTCTCTAAGG-3‘
C03[2]	$(TTCG)_{13}$	134 - 167	C03-fwd: 5‘-ACCGTGTAAATCCAGTCGC-3‘ C03-rev: 5‘-GTCGCCGTGCGGAATAATG-3‘
C06[1]	$(GCTT)_{17}$	294 - 315	C06-fwd: 5‘-TTCCGCGCAACAGAAATCC-3‘ C06-rev: 5‘-TAGGCACGTAACGGGAGTG-3‘
D11[4]	$(TCG)_{30}$	204 - 243	D11-fwd: 5‘-CTGCGTTATACACCATCCGC-3‘ D11-rev: 5‘-ACGAAGGCATTACATACTTGTC-3‘
F05[2]	$(GTGA)_{13}$	205 - 222	F05-fwd: 5‘-ATGCCCGTGTTTCATGCAG-3‘ F05-rev: 5‘-GCATATATTTCGAGGGCGGTC-3‘
F09[2]	$(GTCGA)_{8}$	174 - 200	F09-fwd: 5‘-TCGATGAGGTGATCTCGGG-3‘ F09-rev: 5‘-TCTGCTTCGGATTACGGAAAG-3‘
F11[1]	$(GTCA)_{16}$	169 - 190	F11-fwd: 5‘-TCCTTCGCCCTCGATAGTG-3‘ F11-rev: 5‘-TTCCCGATGAGTTTCACGC-3‘
G06[4]	$(GAGCC)_{10}$	245 - 270	G06-fwd: 5‘-GGGATGCGCACCATAAACC-3‘ G06-rev: 5‘-GAACGAGGGAAACGGGATG-3‘
H08[3]	$(GCGAT)_{9}$	180 - 202	H08-fwd: 5‘-ATCGTCCTCGCTCTGGAAG-3‘ H08-rev: 5‘-TCGATTCGCTCCGAAATGC-3‘

CB2 (Jermiin & Crozier 1994), which produced a 750 bp fragment of *cytb.* These sequences were also compared to those obtained by the other primer pairs. All multiply covered sequences were identical.

PCR was carried out in 25 µl volumes containing PCR-Buffer with a final $MgCl_2$ concentration of 3.1 mM, 0.09 mM dNTPs, 0.5 µM of each primer and 0.5 U *Taq* (Peqlab, Erlangen, Germany). DNA amplification was performed with a Mastercycler EP S (Eppendorf, Hamburg, Germany) and consisted of 3 min initial denaturation at 94 °C, followed by 35 cycles with 30 s denaturation at 92 °C, 30 s annealing at 46 °C – 47 °C, 1 min elongation time at 68 °C and a final 10 min elongation step at 72 °C. From elongation step 11 onwards, the elongation time was expanded stepwise from 1 min to 2 min.

PCR products were loaded on an agarose gel to check for correct product size and potential unintended byproducts. Afterwards, products were cleaned using ExoSap (ThermoFisher Scientific) and sequenced on a capillary sequencer (ABI3730; Life Technology, Darmstadt, Germany) at the Senckenberg Biodiversity and Climate Research Centre (BIK-F) in Frankfurt / Main (Germany).

Sequences were aligned using ClustalW and checked manually. The mitochondrial genome of *Solenopsis richteri* Forel 1909 with annotated CDS was used to align all coding regions. The mtDNA genetic code in insects differs slightly from the standard genetic code. Since most phylogeny programs cannot account for it, the respective codons had to be changed. In detail, the *Drosophila* code codes differently for Ser and Met and, more importantly, has one stop codon less (“UGA” codes for Trp instead of *). Codons at twelve positions had to be changed from “UGA” to “UGG” across *ND6* and *cytb* to run codon models. This reduced the variance at four out of these twelve positions (because the third codon position was already “G” in some individuals), which is considered to be minimal compared to the high variance within the alignment. The final alignment of the 32 ingroup individuals comprises 2280 bp with 118 variable sites (129 mutations) and consists of 23 haplotypes.

Phylogenetics models were run in MrBayes 3.2 (Ronquist & al. 2012). Three different runs with three chains were started for each analysis and the likelihood values checked for convergence to identify the minimum number of burn-in generations. The final burn-in period was set at twice the number of steps at which no further improvement in the likelihood could be observed, and the analyses were run for 300,000 to 5,000,000 generations. Phylogenies were calculated for *ND6* and *Cytochrome b* separately, as well as for the combined data set. To find the model fitting the data best, we applied nucleotide substitution models (identified by MrModeltest, Nylander 2004) as well as codon models. Those two models were run with equal parameters for *ND6* and *cytb* and compared to models which allowed the parameters to vary between the two partitions. The partitioned codon model was additionally compared to a strict molecular clock model, which forced the substitution rate to be equal along all branches. In a last step we applied the multi-species coalescence model (Edwards & al. 2007, Liu & Pearl 2007) to account for the found incongruence between the morphology-based species tree and the gene trees.

Estimates of population differentiations were calculated with DnaSP (Rozas & Rozas 1999) using the distance measure of Nei (1987) with Jukes and Cantor correction.

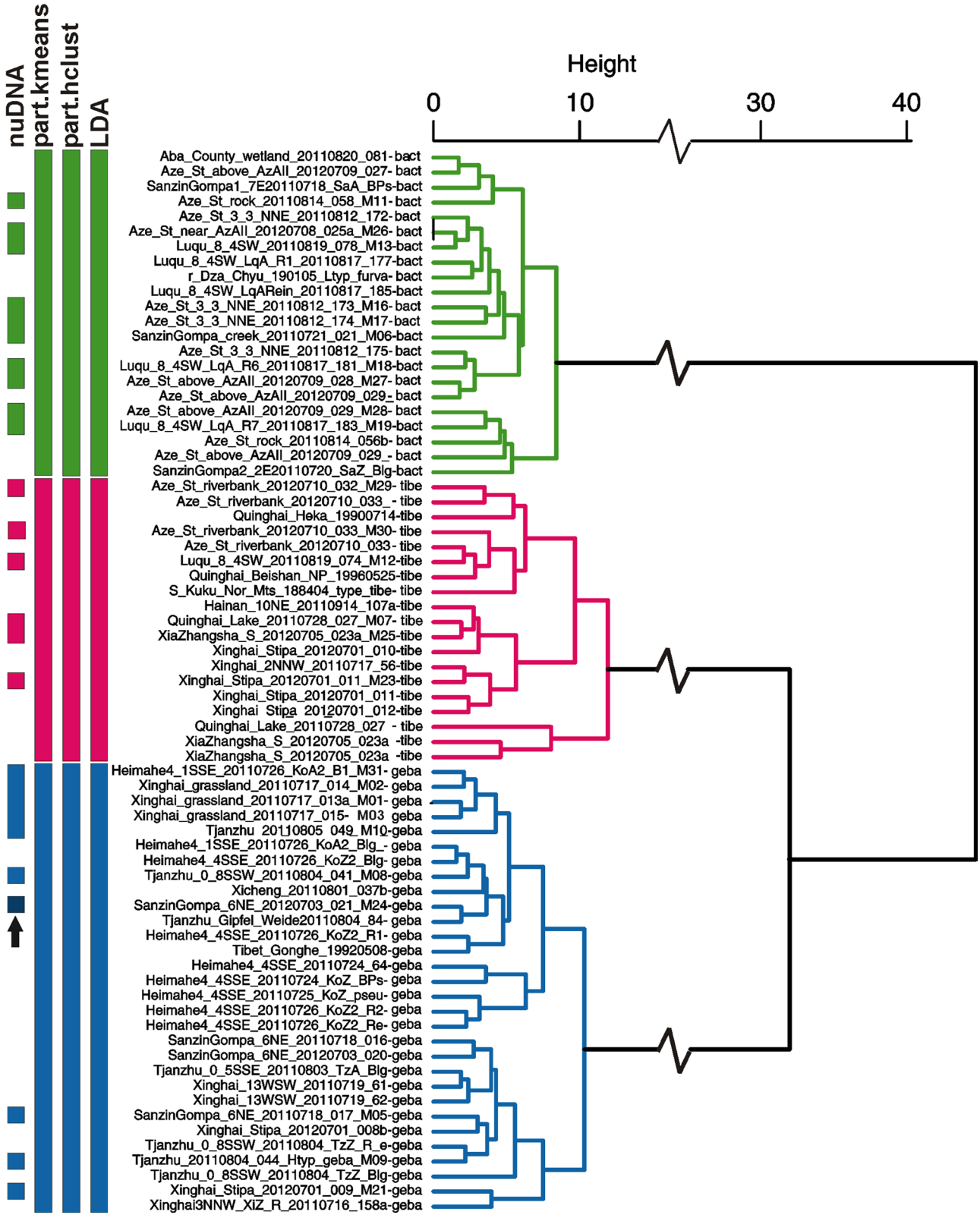

Fig. 1: NC-Ward clustering of worker nest samples of *Myrmica bactriana* Ruzsky, 1915 (upper, green branch), *M. tibetana* Mayr, 1889 (middle, red branch) and of *M. gebaueri* sp.n. (lower blue branch), considering nine morphometric characters. Bars indicate the classification by nuDNA (microsatellites), the partitioning algorithms part.kmeans and part.hclust based on recursive thresholding, and linear discriminant analysis. The arrow points to the sample placed in the microsatelite analysis intermediate between *M. gebaueri* and *M. tibetana*. A direct sample-by sample comparison with the microsatellite topology given in Figs. 3 and 4 is possible with the "M01"…"M30" strings at the end of the labels. The tree topology of mtDNA (Fig. 6) differs very strongly. For comments on the taxonomic value of mtDNA barcoding see the main text.

Microsatellite analyses

Microsatellite markers were developed by the company Starseq (Mainz, Germany) based on an Illumina-Miseq DNA library. After shearing, end-repairing, A-tailing and ligating to TruSeq adapters 100 ng genomic DNA of *Myrmica tibetana*, the library was amplified in eight cycles. Then the library was selected for a mean of 650 bp corresponding to 530 bp internal sequence length and then sequenced for 300 bp in "paired-end" module in one Illumina Miseq run. This resulted in 42 million "paired-end-reads" (12.7 Gb) in total. The overlapping "paired-end-reads" were assembled using FLASH (Magoc & Salzberg 2011) and screened for microsatellite motifs. Initially, 96 loci were selected, for which primers were designed. The 25 µl reaction mixture for amplifications contained 1 µl template DNA (10 ng), 1 µl of each primer (10 µM), 2 µl dNTPs (2.5 mM), 5 µl 5x PCR buffer (Promega, Mannheim, Germany), 2 µl $MgCl_2$ (25 mM), and 0.1 µl G2GoTaq-HotStart polymerase (1.25 U; Promega) and 12.9 µl aqua bidest, and PCR was performed under the following temperature cycles: initial denaturation for 3 min at 95 °C followed by 34 cycles each consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were analysed using QIAxcel by Qiagen (Hilden, Germany) and 48 out of 96 reactions yielded distinct products of the respective expected size. Variability of the loci was tested by comparing PCR products of several individuals of *Myrmica tibetana* resulting in 11 suitable loci (Tab. 1). These loci were cross-amplified in *M. gebaueri* and *M. bactriana* and yielded also suitable results.

We performed the following multiplex PCR reactions (primer combinations see Tab. 1) based on the method of Schuelke (2000) in a total volume of 11.5 µl: 1 µl template DNA (10 ng), 1.25 µl of fwd primer (2 µM), 1.25 µl of rev primer (8 µM), 0.3 µl labeled M13 primer (2 µM), 2 µl dNTPs (2 mM), 1.25 µl S PCR buffer (Peqlab), 0.63 µl Enhancer (Peqlab) and 0.25 µl *Taq* polymerase (1.0 U; Peqlab). The PCRs were performed in an Eppendorf Mastercycler EP S programmed for 15 min at 94 °C followed by 34 cycles of 30 s at 94 °C, 30 s at 59 – 63 °C (see Tab. 1) and 60 s at 72 °C and a final extension for 30 min at 72 °C. Fragments were analysed on an ABI3730 sequencer using the size standard LIZ-500 (Life Technology) and scored with the software Peak Scanner v. 1.0 (Thermo Fisher Scientific).

We detected 5 - 10 alleles per locus and the final data set contained 26 samples and 89 alleles (5.1% missing values; supplementary information SI1, as digital supplementary material to this article, at the journal's web pages). The genotype data were transformed into Bruvo-distances, which incorporate mutational distances between alleles by including repeat motifs (Bruvo & al. 2004) using the package POLYSAT (Clark & Jaseniuk 2011) running under R environment (R Core Team 2016). Based on Bruvo distances we performed Principal Coordinate Analysis (PCoA; based on square-rooted distances) in R using the VEGAN v. 2.3.5 (Oksanen & al. 2015) and Neighbor-Net analysis using Splitstree v. 4 (Huson & Bryant 2006). Bayesian clustering was computed with the program Structure v. 2.3.4 (Pritchard & al. 2000; Falush & al. 2007) for co-dominant markers applying admixture model with correlated allele frequencies among populations. All runs of Structure were done without including geographic or morphological information. We analysed 1,000,000 generations after burn-in

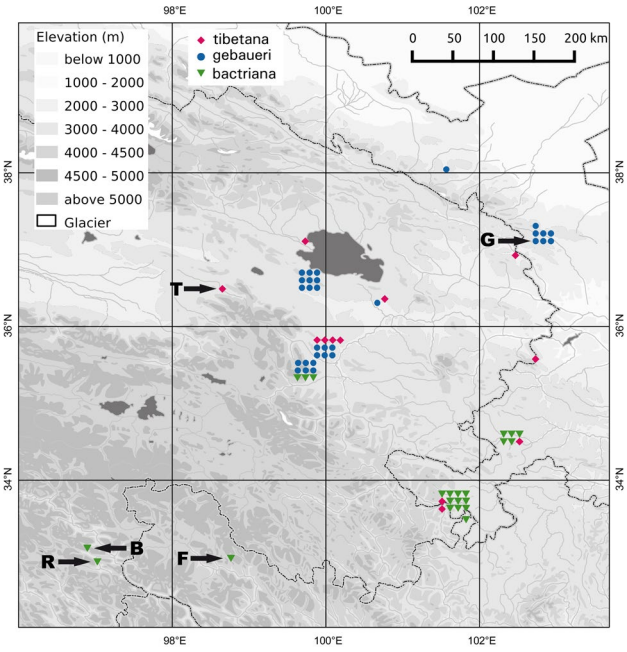

Fig. 2: Sampling sites of *Myrmica bactriana* Ruzsky, 1915 (green triangles), *M. tibetana* Mayr, 1889 (red rhombs), and of *M. gebaueri* sp.n. (blue dots) on the Tibetan Plateau. Coordinates are slightly manipulated to visualize the number of samples per site. Arrows point to type localities; G = *M. gebaueri* sp.n., T = *M. tibetana*; R = *M. ruzskyana* Radchenko & Elmes, 2010; B = *M. bactriana*, F = *M. furva* Ruzsky, 1915.

(500,000) for 10 replicates of models consisting of 1 to 6 clusters (K value). According to the method described by Evanno & al. (2005) the optimal number of clusters for the data set was three clusters (K = 3). The result of this model (K = 3) was graphically displayed using Distruct v.1.1 (Rosenberg 2004).

Results and discussion

Phenotypic clustering is convincing in explorative and supervised approaches

Myrmica tibetana Mayr, 1889, its cryptic sibling *M. gebaueri* sp.n. (formally described below) and *M. bactriana* Ruzsky, 1915 were convincingly demonstrable by NC clustering – the argumentation for the taxonomic naming of these clusters is provided below. Considering all 18 characters unselectively – the size indicator CS, the shape characters CL / CW_{950}, SL / CS_{950}, EYE / CS_{950}, FL / CS_{950}, FR / CS_{950}, PEW / CS_{950}, PPW / CS_{950}, PEH / CS_{950}, PEL / CS_{950}, SPBA / CS_{950}, SPTI / CS_{950}, SP / CS_{950}, MetL / CS_{950}, MetSp / CS_{950}, PoOc / CL_{950}, FL / FR_{950} and the setae character PPHL / CS_{950} – all three methods of exploratory data analyses showed three clear clusters with only two samples disagreeing in classification. If the latter were run as wild-cards in a three-class LDA to determine their final species hypothesis, they were allocated with posterior probabilities of p = 0.961 and p = 1.000. As result, NC-part.kmeans showed 0% deviation from the final species hypothesis whereas NC-Ward and NC-part.hclust misplaced one sample each, meaning 1.6% error (dendrogram not shown). These data show that the phenotypic classification was very strong already in the first run of exploratory data analyses considering all characters

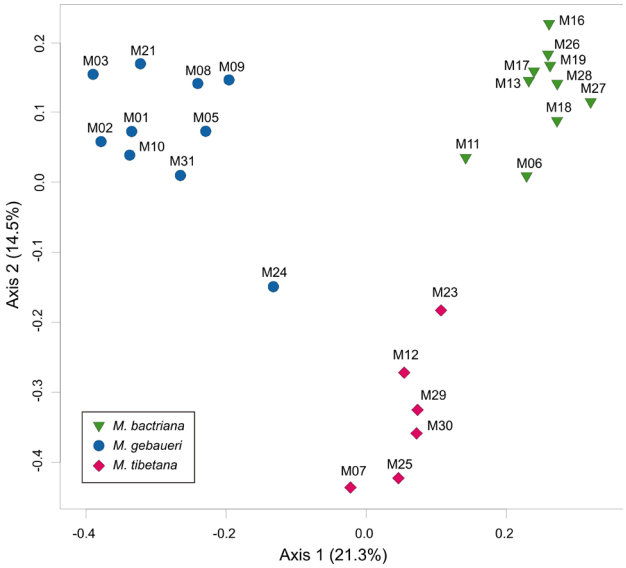

Fig. 3: Principal Coordinate Analysis of microsatellite data of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889, and of *M. gebaueri* sp.n. based on Bruvo distances presenting the first two axes.

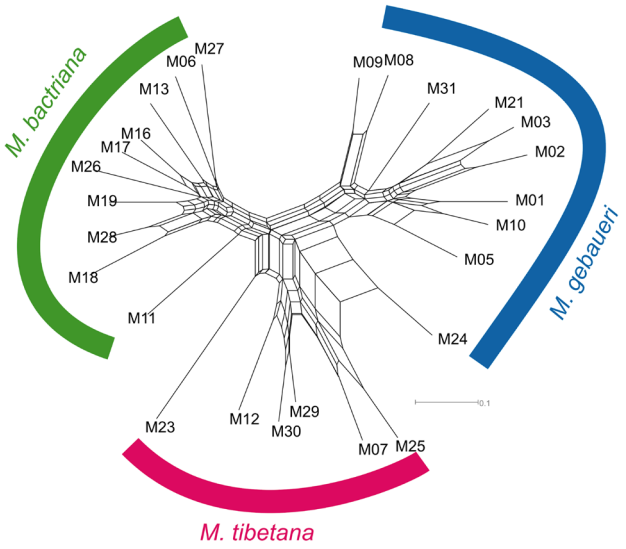

Fig. 4: Neighbor-Net analysis of microsatellite data of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889, and of *M. gebaueri* sp.n. based on Bruvo distances.

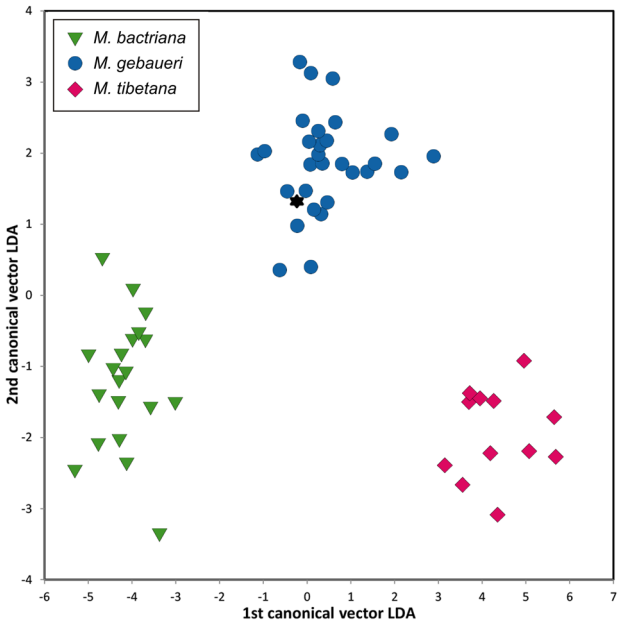

Fig. 5: Linear discriminant analysis (LDA) of 18 morphometric characters of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889 and of *M. gebaueri* sp.n. The black star represents sample M24 which was placed in the microsatelite analysis intermediate between *M. gebaueri* and *M. tibetana* and was run in the LDA as wild-card.

unselectively. The power of the applied NC-clustering methods becomes obvious if one considers that 54% of individuals are placed in the interspecific overlap range of the most discriminative character to separate the cryptic species *M. gebaueri* sp.n. and *M. tibetana* (EYE / CS).

To improve the separation, we ran a stepwise LDA reducing the number of characters to nine: EYE / CS_{950}, FL / CS_{950}, PPW / CS_{950}, PEL / CS_{950}, SPBA / CS_{950}, SPTI / CS_{950}, SP / CS_{950}, FL / FR_{950} and PPHL / CS_{950}. Using these characters, the classification of the 62 samples by all three data analyses was 100% coincident and no sample was misplaced (Fig. 1). The character reduction provided the favorable situation that the number of individuals in the smallest class (n = 45 in *Myrmica tibetana*) was 5fold larger than the number of considered characters. Under this condition, the LDA and the leave-one-out-cross-validation LDA (LOOCV-LDA) confirmed the three-species classification in 178 individuals with an error of 0% and 0.6% respectively. If all individuals of the type samples were run as wild-cards in a LDA, the posterior probabilities were 1.000 in the lectotype of *M. tibetana*, 0.996 in the whole type series of *M. tibetana*, 1.000 in the holotype of *M. gebaueri* sp.n., 0.998 in the whole type series of *M. gebaueri* sp.n. and 1.000 in both type specimens of *M. furva* which were allocated to the *M. bactriana* cluster.

This very clear phenotypic clustering, the sharing of sympatric areas by all three entities and their syntopic occurrence at several sites (Fig. 2) clearly indicate separate species with significant reproductive barriers. There is also no indication for phenotypically mixed nests which excludes to explain *Myrmica gebaueri* sp.n. as intraspecific polymorphism of *M. tibetana* (Seifert 2016).

Microsatellite analysis confirms morphological classification

Analyses based on microsatellite data support the morphological discrimination into three species. The first two axes of the Principal Coordinate Analysis explain 35.8% of the variance in the data set and separate clearly between *Myrmica bactriana, M. gebaueri* and *M. tibetana* (Fig. 3). However, the sample M24 of *M. gebaueri* is placed intermediate between the remaining samples of *M. gebaueri* and *M. tibetana*. The situation in the Neighbor-Net analyses (Fig. 4) is likewise: A basically clear clustering into three species in agreement with morphology but an intermediate position of M24. We consider this result to be caused by the low sample size of *M. tibetana* causing instability in microsatellite analyses rather than to indicate a hybrid or

introgression of gene material. Morphological data clearly contradict a hybrid identity of M24. It has been repeatedly shown that NUMOBAT data indicate ant hybrids when the parental species are sufficiently separated in the vectorial space (Seifert 1984, 2006, Kulmuni & al. 2010, Seifert & al. 2010, Steiner & al. 2011, Bagherian Yazdi & al. 2012). In cases when the parental species are less clearly separable, the hybrid cluster may be close to one parental cluster or may merge with it (Seifert 2006). Yet, there is no case known where a hybrid sample is placed close to the centroid of one parent. If the three workers of sample M24 are run as wild-card in a three-class LDA considering all 18 phenotypical characters both individuals and sample mean are placed very near to the centroid of the *M. gebaueri* cluster (Fig. 5) with posterior probabilities of 1.000 in any case. A further argument for the species identity of M24 is provided by analysis of mtDNA: M24 is placed in the (paraphyletic) mtDNA tree within a branch only composed of *M. gebaueri* samples (Fig. 6).

Indication by mtDNA barcoding is in strong conflict with true species identities

The use of mitochondrial DNA as a leading tool in alpha-taxonomy is most problematic as it was already shown in the classic meta-analysis of 323 genera of Eumetazoa presented by Funk & Omland (2003). Given that other sources of error such as NUMTs (Bensasson & al. 2001) are excluded, the high frequency of paraphyly remains the biggest problem (e.g., Nichols 2001, Sota & Vogler 2001, Besansky & al. 2003, Funk & Omland 2003, Shaw 2003, Ballard & Whitlock 2004, Kocher 2004, Heinze & al. 2005, Hurst & Jiggins 2005, Lorenz & al. 2005, Mendelson & Shaw 2005, Meier & al. 2006, Wells & al. 2007).

The situation is similar in ants. Considering studies where mtDNA indication is controlled by reproducible and testable data sets of NUMOBAT and / or nuDNA, the error of barcoding on the alpha-taxonomic level ranges from 6% in *Tapinoma* (Seifert & al. 2017a) and 7% in *Cardiocondyla* (Seifert & al. 2017b) to 15% in the *Formica rufa* group (Seifert & Goropashnaya 2004), 17% in *Tetramorium* (Wagner & al. 2017), 19% in Neotropical *Linepithema* species (Wild 2009), and 23% in African *Cataglyphis* (Knaden & al. 2005). Considering studies with idiosyncratic morphology-based taxonomy as supervising system, mtDNA barcoding errors appear to be in the same range – e.g. in the genera *Anochoetus* and *Odontomachus* (Fisher & Smith 2008) and *Solenopsis* (Shoemaker & al. 2006). There is no integrative study known for ants, in which all disciplines were run in a controlled and testable mode, where mtDNA barcoding errors were below the range delimited above.

Taking mtDNA indication as final truth for the alpha-taxonomic structure of the Tibetan *Myrmica* studied here and accepting only nodes with bootstrap supports > 0.99, we would suppose six (or perhaps eight) instead of three species (Fig. 6). Relating a 6-species hypothesis based on mtDNA to the 3-species hypothesis achieved above by integrative taxonomy of NUMOBAT and nuDNA data, we have a minimum barcoding error of 24% if only the smaller of the deviant branches are considered as wrong and a bigger error if the larger deviant branches were wrong. The resulting average error of 16% of now seven studies in ants where mtDNA barcoding was controlled by reproducible and testable data sets of NUMOBAT and / or nuDNA shows the magnitude of the problem. These ant data add to the burden of evidence against the application of mtDNA barcoding as a leading tool in alpha-taxonomy.

In the particular case of the *Myrmica tibetana* species complex, we have no really strong data to conclude on the possible reasons for the mismatch between mtDNA barcoding and true species identities. A check, if different species shared mtDNA clades attributable to geographic spots in Tibet, was fully negative. From this point of view, incomplete lineage sorting appears as a less likely explanation for mismatches than occasional ancient hybridization with introgression of mtDNA. The latter issue has recently got a new, extreme component revealed by observations in *Formica* ants: there is virtually a selection favouring a mismatch of mitochondrial and nuclear DNA after a hybridization event and this selection acts in both directions instead of following the usual unidirectional pattern (Beresford & al. 2017). This situation may basically occur in any organisms with haplo-diploid sex determination.

Estimation of divergence times by mtDNA

There are strong methodological problems with datings of divergence times (see Takahata 2007 and references therein, Wilke & al. 2009). Furthermore, there is no fossil-based dating in *Myrmica* covering the range of the last 30 million years – Jansen & al. (2010) calibrated their *Myrmica* topology by fossil records dating back to 92 and 44 Ma. In the absence of ant-specific datings we used 1.2% nucleotide substitutions per Ma estimated for protostomians with the GTR model (Wilke & al. 2009). This certainly is under risk of a considerable error.

Based on the mean log likelihood values, variation in the data set was best explained by a codon model (GTR) with different parameters for the *ND6* and *Cytochrome b* partition, respectively. The partitioned codon model was clearly better than the same model with a strict molecular clock (delta log likelihood = 25) which is most likely due to a considerably lower evolutionary rate of the *Myrmica tibetana*-clade. The two phylogenies based on *ND6* and *cytb* separately showed the same topology as the data set combining the two genes and there was an identical tree topology when the analysis was run in BEAST (Drummond & al. 2012).

In contrast to NUMOBAT and nuDNA indication, Fig. 6 suggests a clear separation of a *Myrmica tibetana* clade (Tibe clade) from a *M. bactriana* / *M. gebaueri* sp.n. clade (BactGeba clade). The average number of nucleotide substitutions per site between the Tibe clade and the BactGeba clade was 10% in the GTR model which would translate into a divergence time of about 8 Ma when the 1.2% per Ma estimate should apply. The BEAST analysis suggested a range of 4.5 to 10% nucleotide substitutions corresponding to about 4 to 8 Ma. The Tibe clade and the four subclades of the BactGeba clade (Bact1, Bact2, Geba1 and Geba2) are "clean" – i.e., each of the five clades always contains only a single species classified by NUMOBAT and microsatellites. As most likely explanations for this type of paraphyly appear both ancient hybridization and incomplete lineage sorting when *M. bactriana* and *M. gebaueri* sp.n. split off. The fact that each of the four paraphyletic subclades contains only a single species may indicate that there was no crossbreeding or introgression event between *M. bactriana* and *M. gebaueri* sp.n. for a rather long evolutionary period.

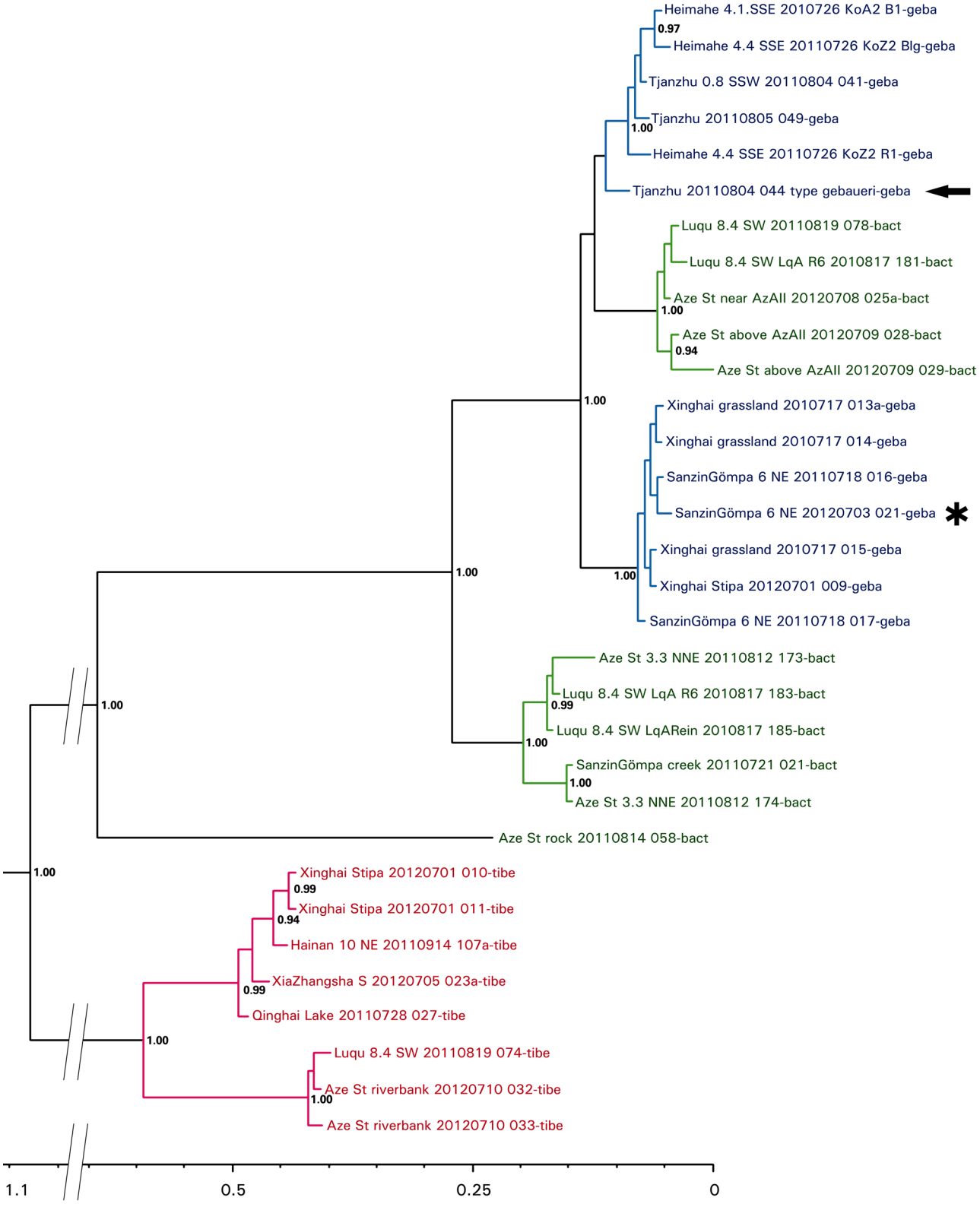

Fig. 6: mtDNA phylogeny of the *Myrmica tibetana* species complex based on the *cytb* and parts of *ND6* mitochondrial genes. Samples of *M. bactriana* Ruzsky, 1915 (in green), *M. gebaueri* sp.n. (in blue) and of *M. tibetana* Mayr, 1889 (in red). Support values (Bayesian posterior probabilities) < 0.90 are not shown. The arrow points to the type sample of *M. gebaueri* sp.n. and the asterisk marks the sample M24 which was placed in microsatellite analysis intermediate between *M. tibetana* and *M. gebaueri*.

Rapid speciation and development of cryptic species in the geographic region dealt with here has often been considered to be a consequence of a rapid rather recent uplift of the Tibetan Plateau (e.g., Liang & al. 2015, Liu & al. 2013, Lu & al. 2014, Wu & al. 2011). The strongest uplifts, generating the present shape of the Qinghai-Tibetan Plateau, were supposed to have occurred between 3.6 and 0.15 Ma Before Present (BP) and to have increased the mean altitude of the Plateau from 1000 to 4400 m (Li & Fang 1999). Yet, strongly opposing this extreme view, multiple arguments (reviewed in Renner 2016) rather indicate that a height of 4000 m has already been achieved by 40 Ma. Renner described a situation based on few outdated geological papers (such as Li & Fang 1999) as follows: "Biogeography of the Tibetan Plateau thus currently appears to be in a self-created bubble that encloses hundreds of authors and referees". As much further research has to be done on the issue, it is highly speculative, if not decisively wrong, to discuss speciation in the *Myrmica tibetana* complex within the context of a rapid Pliocene uplift scenario.

One sample of *Myrmica bactriana* from Aze Station, 2011.08.14, field number No 058 (= M11 in nuDNA data) showed a mtDNA sequence clearly different from the other conspecific samples (Fig. 6). We can offer no *a priori* explanation for this outlier. The sequence contains no frame shifts and no stop codons. We got identical sequences over 1370 bp across three genes (*ND6*, tRNASer, *cytb*) with three primer pairs. All workers from this nest sample showed no morphological abnormalities. This refers both to the complete nest series checked by subjective assessment and to the three workers investigated in the multivariate analyses. They were positioned near to the species' centroid.

How do our time estimates fit to the concept on *Myrmica* evolution of Jansen & al. (2010) in which two nodes were fixed by very ancient palaeochronological data? In their molecular phylogeny of Holarctic species, these authors identified a distinct monophyletic clade which they called the *M. rubra* group. Their material of this group contained samples of *Myrmica rubra* (Linnaeus, 1758), *M. ruginodis*, *M. kotokui* Forel, 1911 and *M. arisana* Wheeler, 1930, but they did not sample a species of the *M. tibetana* complex. The relatedness of the four species of the *M. rubra* group sensu Jansen & al. (2010) is confirmed by morphology: in the worker caste they share a produced, angular-convex clypeus without a median notch and a slender, moderately bent scape base without edges or carinae. Their males resemble in having a long scape with a slender, moderately bent base without edges or carinae (see also Radchenko & Elmes 2010). These are just the characters found in *M. tibetana, M. bactriana* and *M. gebaueri* sp.n. indicating a close relatedness with the *M. rubra* group sensu Jansen & al. (2010). Considering the findings of Radchenko & al. (2007) who dated the first *Myrmica* from Baltic and Saxonian amber back to 44.1 Ma BP, Jansen & al. (2010) estimated the beginning of radiation in the genus *Myrmica* back to the Eocene (41 Ma) and that of the *M. rubra* group to the Miocene (11 Ma). Our estimates of a divergence time of the *M. tibetana* complex from other members of the *M. rubra* group of about 16 Ma would indicate an earlier splitting.

Assessment of phylogenetic relatedness by the three indicators – morphology, nuDNA and mtDNA appears controversial. NC-Ward-clustering of morphology (Fig. 1) and NC-UPGMA clustering (not shown) suggest a sibling species relation between *Myrmica gebaueri* sp.n. and *M. tibetana* but mtDNA suggests a higher relatedness of *M. gebaueri* sp.n. and *M. bactriana*. In the morphological data set, a single character is responsible that *M. tibetana* and *M. gebaueri* sp.n. emerge from a common node in the dendrograms: They share a strong extension of frontal lobes (Fig. 7) and just this character is the most powerful discriminator of the two from *M. bactriana*. Yet, a summaric comparison over all characters (Tab. 2) shows that *M. gebaueri* sp.n. and *M. bactriana* show no significant differences in 47% of the characters whereas this figure is only 24% when *M. gebaueri* sp.n. and *M. tibetana* are compared. Thus it seems possible that metric characters as they are used here may lead to wrong genealogies in dendrograms of a group of cryptic species – the more as some of these characters are adaptive and subject to convergent evolution.

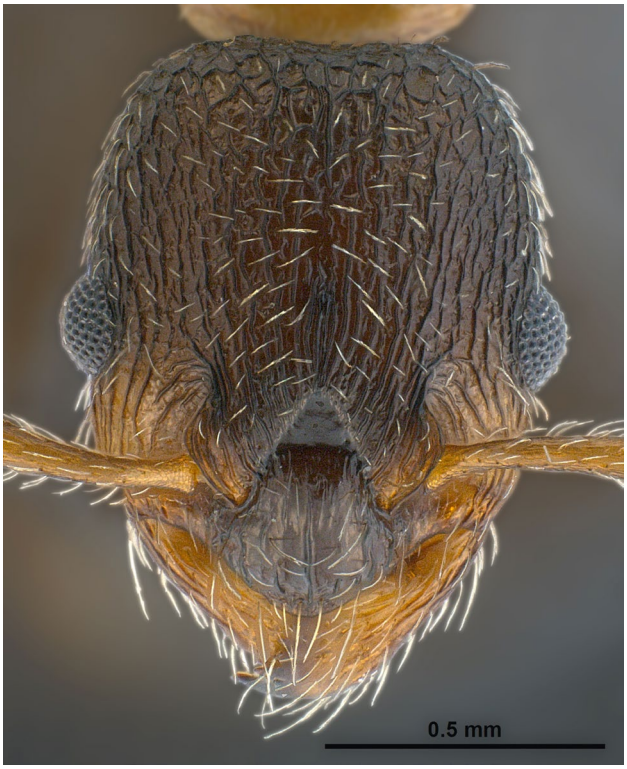

Fig. 7: Head of holotype of *Myrmica gebaueri* sp.n. in dorsal view.

Consideration of synonymies

Type samples of three taxa were available for the multivariate analyses and each of these was allocated to a different cluster. Because we introduce here a new species it must be asked if there are possible synonyms among described Palaearctic taxa of which types were not available. We found six candidates. The first two are *Myrmica bactriana* Ruzsky, 1915 [determined by Radchenko & Elmes (2010) as senior synonym of *M. furva* Ruzsky, 1915] and *M. ruzskyana* Radchenko & Elmes, 2010. The drawings of the lectotypes of *M. bactriana* and *M. ruzskyana* show only very slightly diverging frontal lobes: The ratio FL / FR is 1.070 and 1.080 respectively, both have a wedge shaped anterior clypeal margin, rather short scapes with a slender moderately curved basal part and numerous semierect setae, short and acute propodeal spines, a rather low petiole showing in profile a rounded node and no angular elements. The characters of

Fig. 8: Mesosoma of holotype of *Myrmica gebaueri* sp.n. in dorsal view.

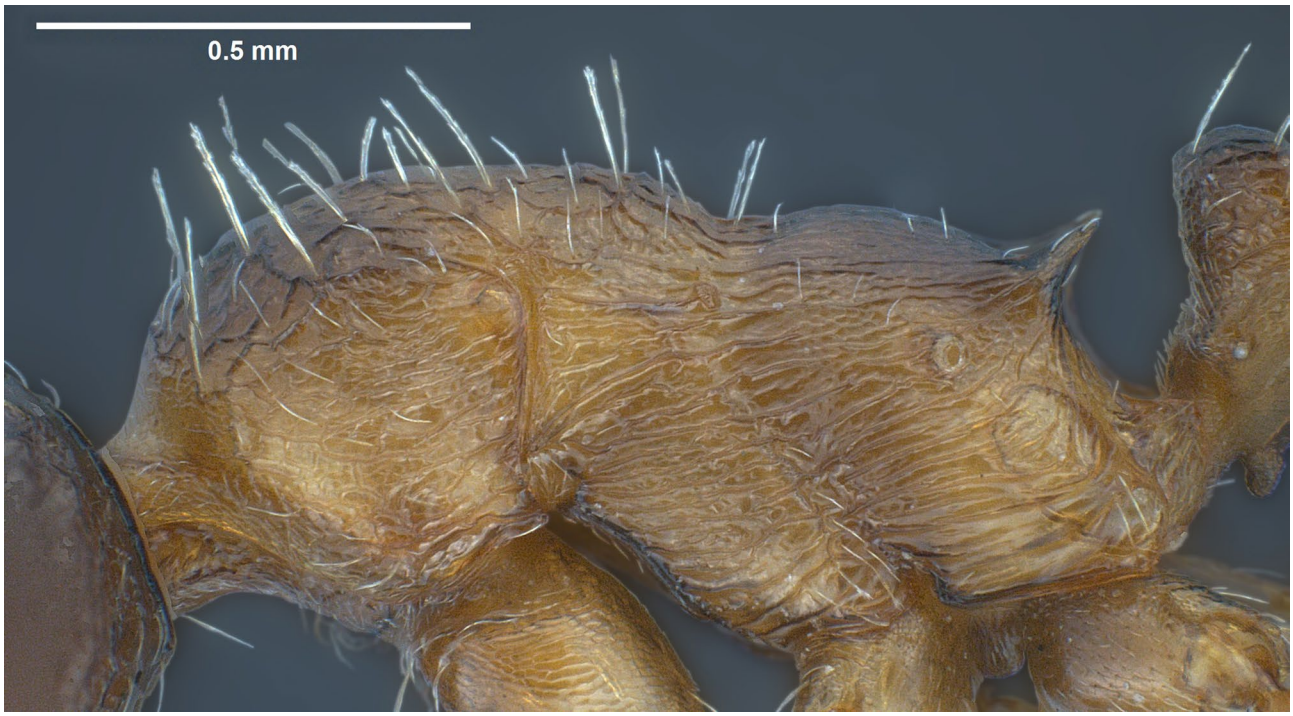

Fig. 9: Mesosoma of holotype of *Myrmica gebaueri* sp.n. in lateral view.

the investigated type sample of *M. furva* coincide with this diagnosis and FL / FR is similarly low: 1.083 and 1.115. There is no significant difference visible between these three taxa and their type localities are found in just the same region (Fig. 2): The basin of the river Yangtse around the present town of Yushu. The type localities of *M. bactriana* and *M. ruzskyana* are nearly syntopic and that of *M. furva* is perhaps some 170 km east. Radchenko & Elmes (2010), in separating *M. bactriana* and *M. ruzskyana,* presented the following arguments. "*M. bactriana* is very similar to *M. ruzskyana*, differing only by its distinctly longer scape ($SI_2 \geq 0.93$ vs. ≤ 0.91) with more abundant suberect hairs, and it is quite possible this represents different populations of the same species." We found that scape pilosity and length differed considerably within our *M. bactriana* material and do not have taxonomic significance. Arithmetic

mean, standard deviation, minimum and maximum of the scape length index SI_2 were 0.928 ± 0.23 [0.875, 0.992] in 52 measured workers of *M. bactriana* and several nest samples contained workers with both $SI_2 \geq 0.93$ and ≤ 0.91. Furthermore the NC-Ward dendrogram (Fig. 1) does not indicate a clear morphology-based substructuring with the *M. bactriana* clade and NC-part.hclust and NC-part.kmeans could not resolve subclusters. These data multiply to a high probability that *M. bactriana, M. furva* and *M. ruzskyana* belong to the same species and we follow Radchenko & Elmes (2010) in determining *M. bactriana* as the senior synonym.

The ratio FL / FR varied in the types of *Myrmica bactriana*, *M. furva* and *M. ruzskyana* in the narrow span between 1.070 and 1.115 whereas it ranged 1.162 - 1.319 in 45 workers of *M. tibetana* and 1.151 - 1.275 in 81 workers of *M. gebaueri* sp.n. This argument alone excludes a synonymy of *M. tibetana* and *M. gebaueri* sp.n. with *M. bactriana* and its synonyms.

The angularity and much stronger divergence of the frontal lobes also excludes a synonymy of the Himalayan *Myrmica smythiesii* Forel, 1902 [FL / FR of the lectotype 1.085 according to drawing in Radchenko & Elmes (2010)], *M. fortior* Forel, 1904 [FL / FR of a paralectotype 1.046 according to a photo from www.AntWeb.org, specimen CASENT0904090] and *M. wittmeri* Radchenko & Elmes, 1999 [FL / FR of the lectotype 1.060 according to drawing in Radchenko & Elmes (2010)]. Furthermore, the Himalayan species live in a very different climatic context compared to the Tibetan Plateau: mean annual air temperatures are by 10 – 15 °C higher and annual precipitations 2 - 3fold larger.

The 6th possible synonym of *Myrmica gebaueri* sp.n. – *M. tenuispina* Ruzsky, 1905 – was described from Western Tian Shan. It is similar to the species of the *M. tibetana* complex in overall body size, sculpture, shape of scape, clypeus and petiole and it inhabits a comparable montane to subalpine grassland habitat. A synonymy can be clearly excluded because the three measured worker specimens of the lectotype series of *M. tenuispina* showed a scape and spine length much larger than known for any specimen of the *M. tibetana* complex. The index SL*SP / FR was

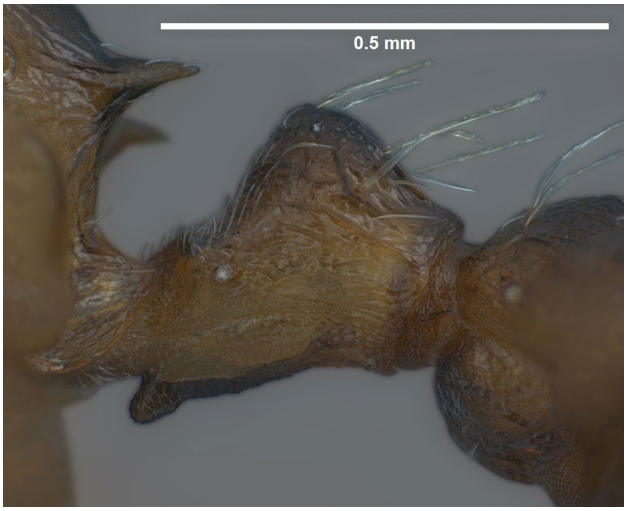

Fig. 10: Petiole of holotype of *Myrmica gebaueri* sp.n. in lateral view.

0.345 ± 0.038 [0.234, 0.440] in 178 workers of the *M. tibetana* complex but 0.679 ± 0.022 [0.653, 0.693] in the three *M. tenuispina* types and 0.610 ± 0.067 [0.526, 0.708] in 19 workers of *M. tenuispina* from Tian Shan.

***Myrmica gebaueri* sp.n.**

Etymology: Named after the German naturalist Axel Gebauer who made several expeditions to the Tibetan Plateau and was the first to collect this species in 1992.

Type material: Holotype labeled "CHI: 37.1852° N, 102.7844° E, Tianshu station-1.2 S, 2939 m moist pasture, under stone R.Schultz 2011.08.04-044" and "Holotype *Myrmica gebaueri* Seifert, Ritz & Schultz"; two worker paratypes on a different pin and 27 worker paratypes stored in ethanol – all from the same nest sample and with equal collecting data label as the holotype; all material stored in SMN Görlitz.

Description: Worker (Figs. 7 - 11, Tabs. 2 - 3, all morphometric ratios given in the following description are

Fig. 11: Left scape of holotype of *Myrmica gebaueri* sp.n. in caudodorsal view.

Tab. 2: Data of worker individuals of the three species of the *Myrmica tibetana* complex given as arithmetic mean ± standard deviation [lower extreme, upper extreme]; i = number of individuals. F values and significance levels p are from an univariate ANOVA and placed between the columns of the species pair compared; the F values of the characters best separating the species are given in heavy type.

	M. bactriana (i = 52)	**ANOVA F, p**	***M. gebaueri* sp.n.** (i = 81)	**ANOVA F, p**	***M. tibetana*** (i = 45)
CS [µm]	920 ± 37 [845, 1000]	0.00 n.s.	919 ± 37 [809, 1012]	33.16 0.000	960 ± 42 [886, 1059]
CL/CW	1.136 ± 0.017 [1.101, 1.172]	3.86 n.s.	1.129 ± 0.021 [1.070, 1.179]	3.34 n.s.	1.122 ± 0.019 [1.090, 1.169]
SL/CS	0.789 ± 0.017 [0.749, 0.834]	3.12 n.s.	0.794 ± 0.014 [0.750, 0.824]	**21.71** 0.000	0.782 ± 0.012 [0.761, 0.809]
PoOc/CL	0.419 ± 0.007 [0.398, 0.434]	**53.81** 0.000	0.409 ± 0.007 [0.394, 0.433]	3.58 n.s.	0.406 ± 0.010 [0.382, 0.422]
EYE	0.193 ± 0.007 [0.179, 0.209]	3.15 n.s.	0.191 ± 0.005 [0.177, 0.206]	**138.96** 0.000	0.204 ± 0.007 [0.191, 0.216]
FL/CS	0.437 ± 0.009 [0.412, 0.451]	**511.08** 0.000	0.481 ± 0.013 [0.462, 0.507]	**86.95** 0.000	0.502 ± 0.011 [0.478, 0.526]
FR/CS	0.397 ± 0.009 [0.377, 0.415]	4.87 0.029	0.401 ± 0.011 [0.380, 0.430]	0.52 n.s.	0.400 ± 0.011 [0.371, 0.425]
SPBA/CS	0.284 ± 0.012 [0.258, 0.308]	3.21 n.s.	0.281 ± 0.010 [0.262, 0.316]	**47.64** 0.000	0.294 ± 0.012 [0.269, 0.320]
SPTI/CS	0.309 ± 0.022 [0.266, 0.365]	**21.02** 0.000	0.325 ± 0.017 [0.288, 0.369]	**41.01** 0.000	0.302 ± 0.021 [0.269, 0.341]
PEW/CS	0.251 ± 0.009 [0.232, 0.273]	4.43 0.037	0.254 ± 0.011 [0.232, 0.281]	14.40 0.000	0.246 ± 0.012 [0.213, 0.270]
PPW/CS	0.379 ± 0.012 [0.348, 0.403]	**20.42** 0.000	0.390 ± 0.014 [0.359, 0.424]	**50.54** 0.000	0.371 ± 0.015 [0.342, 0.401]
PEH/CS	0.323 ± 0.008 [0.303, 0.348]	0.27 n.s.	0.324 ± 0.010 [0.305, 0.349]	**42.13** 0.000	0.312 ± 0.009 [0.290, 0.326]
PEL/CS	0.456 ± 0.014 [0.424, 0.485]	9.33 0.003	0.463 ± 0.013 [0.437, 0.499]	**50.47** 0.000	0.447 ± 0.012 [0.415, 0.470]
PPHL/CS	0.201 ± 0.010 [0.176, 0.222]	3.53 n.s.	0.206 ± 0.015 [0.121, 0.234]	**33.94** 0.000	0.190 ± 0.013 [0.154, 0.201]
SP/CS	0.180 0.011 [0.158, 0.206]	0.11 n.s.	0.179 ± 0.018 [0.135, 0.223]	**40.33** 0.000	0.159 ± 0.013 [0.125, 0.186]
MetL/CS	0.222 ± 0.009 [0.208, 0.244]	5.98 0.016	0.226 ± 0.010 [0.208, 0.249]	16.46 0.000	0.220 ± 0.007 [0.205, 0.234]
MetSp/CS	0.201 ± 0.013 [0.177, 0.232]	0.26 n.s.	0.200 ± 0.013 [0.171, 0.228]	1.82 n.s.	0.196 ± 0.016 [0.168, 0.227]

arithmetic nest sample means without removal of allometric variance): Most similar to *Myrmica tibetana*. One of the smallest species of the genus (CS 918 µm). Head with a weakly concave to straight posterior margin and strongly convex sides (Fig. 7) and rather elongated (CL / CW 1.129). Postocular distance rather low (PoOc / CL 0.410). Frontal lobes broad and significantly diverging (FL / CS 0.482, FL / FR 1.190), their lateral outline more angulate than convex, usually forming an angle ± 110°, frontal carinae merging with the rugae that surround antennal sockets. Eyes with few microsetae and rather small (EYE / CS 0.191), distinctly smaller than in *M. tibetana* (EYE / CS 0.205). Clypeus in dorsal view of head produced, its anterior margin more angulate than curved, forming an angle of 115 - 125°. Scape moderately long (SL / CS 0.794), with a slender, evenly curved basal part which performs a total bend of ±35° when viewed in the frontal or caudal standard viewing positions (SVP f or c in Seifert & al. 2014; Fig. 11 does not show a fully caudal aspect). Dorsal profile of mesosoma with a strongly convex promesonotal part, a strong metanotal depression and a convex dorsal part of propodeum (Fig. 9). Propodeal spines acute and short but on average longer than in *M. tibetana* (SP / CS 0.179 but 0.155 in the latter), spine axis in lateral view deviating from longitudinal mesosomal axis by 35 - 45°. Spines slightly diverging (Fig. 8): distance of spine base usually smaller than distance of tips (SPBA / CS 0.271, SPTI / CS 0.284) – in *M. tibetana* there is usually no divergence of spines (SPBA / CS 0.282, SPTI / CS 0.263). Central height of propodeal lobe only slightly larger than equal-level height of subspinal excavation (MetL / CS 0.226, MetSp 0.200). Petiole rather low (PEH / CS 0.323) and in lateral view with a concave anterior profile, a rounded dorsum of node and a slightly concave to almost straight caudodorsal profile (Fig. 10). Petiole in dorsal view with rather straight sides, slightly diverging caudad, its width about 65% of postpetiolar width. Setae are present

Tab. 3: Worker nest sample means of RAV-corrected morphometric data in three species of the *Myrmica tibetana* complex given as arithmetic mean ± standard deviation [lower extreme, upper extreme]; n = number of nest sample, i = number of individuals. F values and significance levels p are from an univariate ANOVA; the F values of the characters best separating *M. gebaueri* sp.n. and *M. tibetana* are given in heavy type.

	M. bactriana (n = 20)	**ANOVA F, p**	***M. gebaueri* sp.n.** (n = 30)	**ANOVA F, p**	***M. tibetana*** (n = 12)
CS [µm]	921 ± 32 [855, 977]	1.56 n.s.	918 ± 31 [847, 973]	12.93 0.001	958 ± 38 [907, 1045]
CL/CW (950)	1.134 ± 0.012 [1.108, 1.157]	2.26 n.s.	1.127 ± 0.016 [1.100, 1.153]	1.525 n.s.	1.121 ± 0.012 [1.094, 1.142]
SL/CS (950)	0.786 ± 0.012 [0.761, 0.810]	3.19 n.s.	0.793 ± 0.012 [0.769, 0.822]	11.81 0.001	0.779 ± 0.009 [0.762, 0.796]
PoOc/CL (950)	0.416 ± 0.005 [0.405, 0.423]	21.95 0.000	0.407 ± 0.005 [0.397, 0.422]	0.45 n.s.	0.406 ± 0.008 [0.392, 0.418]
EYE (950)	0.196 ± 0.005 [0.187, 0.204]	3.88 n.s.	0.193 ± 0.004 [0.186, 0.203]	**72.95** 0.000	0.206 ± 0.005 [0.200, 0.215]
FL/CS (950)	0.437 ± 0.006 [0.416, 0.447]	**328.70** 0.000	0.482 ± 0.010 [0.464, 0.502]	**55.08** 0.000	0.505 ± 0.008 [0.494, 0.518]
FR/CS (950)	0.395 ± 0.007 [0.380, 0.407]	4.12 0.048	0.400 ± 0.008 [0.387, 0.417]	0.58 n.s.	0.402 ± 0.007 [0.388, 0.412]
SPBA/CS (950)	0.283 ± 0.010 [0.267, 0.299]	1.20 n.s.	0.280 ± 0.008 [0.261, 0.300]	13.24 0.001	0.291 ± 0.009 [0.271, 0.305]
SPTI/CS (950)	0.303 ± 0.015 [0.279, 0.334]	13.80 0.001	0.318 ± 0.014 [0.289, 0.346]	**24.35** 0.000	0.294 ± 0.017 [0.264, 0.330]
PEW/CS (950)	0.249 ± 0.007 [0.238, 0.263]	3.31 n.s.	0.253 ± 0.006 [0.241, 0.266]	11.38 0.002	0.244 ± 0.011 [0.225, 0.255]
PPW/CS (950)	0.378 ± 0.011 [0.358, 0.404]	11.32 0.002	0.388 ± 0.010 [0.366, 0.405]	18.56 0.000	0.372 ± 0.013 [0.349, 0.392]
PEH/CS (950)	0.322 ± 0.007 [0.309, 0.336]	0.15 n.s.	0.323 ± 0.007 [0.307, 0.334]	**25.91** 0.000	0.311 ± 0.007 [0.299, 0.320]
PEL/CS (950)	0.455 ± 0.011 [0.435, 0.474]	5.16 0.028	0.461 ± 0.009 [0.442, 0.485]	16.60 0.000	0.448 ± 0.011 [0.430, 0.466]
PPHL/CS (950)	0.200 ± 0.008 [0.187, 0.218]	2.82 n.s.	0.205 ± 0.010 [0.170, 0.221]	19.70 0.000	0.190 ± 0.010 [0.175, 0.206]
SP/CS (950)	0.179 0.009 [0.158, 0.194]	0.07 n.s.	0.179 ± 0.015 [0.152, 0.216]	**23.66** 0.000	0.155 ± 0.012 [0.125, 0.167]
MetL/CS (950)	0.223 ± 0.008 [0.212, 0.242]	2.76 n.s.	0.227 ± 0.008 [0.208, 0.243]	8.64 0.005	0.219 ± 0.005 [0.207, 0.226]
MetSp/CS (950)	0.201 ± 0.010 [0.189, 0.220]	0.11 n.s.	0.200 ± 0.010 [0.181, 0.221]	3.40 n.s.	0.194 ± 0.012 [0.174, 0.212]

on all dorsal parts of body and rather long (PPHL / CS 0.206). Vertex moderately strong longitudinally rugose, posterior vertex reticulate; about 16 - 23 rather linear rugae are found between the most approximated parts of frontal carinae. Mesosoma and waist with a weak sculpture in terms of genus *Myrmica*, larger surface areas may be smooth. Dorsum of promesonotum as a rule reticulate-rugose, meso- and metapleuron longitudinally carinulate. Dorsal propodeum weakly carinulate-rugulose, substantial parts of its surface often completely smooth. Dorsum of petiole reticulate-rugulose, central dorsum of postpetiole at lower magnification always appearing smooth and shining, a delicate microreticulum becomes visible at larger magnifications. Whole body usually rather uniformly medium brown with a weak yellowish component and sometimes with a lighter mesosoma.

Distribution and biology: NE Tibet between 35.5 and 38.0° N and 99.8 and 102.8° E (Fig. 2), in altitudes of 2900 - 3500 m. Found on montane to subalpine grassland, usually pastures. Nests in soil, under stones or in grass tussocks. Polygynous.

Systematic position: Based on morphological and genetic arguments, we stated above a close relatedness of the three species of the *Myrmica tibetana* complex to the *M. rubra* group sensu Jansen & al. (2010). Yet, the closest relatives from a morphological point of view most probably are the Himalayan species *M. smythiesii* Forel, 1902, *M. fortior* Forel, 1904 and *M. wittmeri* Radchenko & Elmes, 1999. They resemble the members of the *M. tibetana* complex in the following characters:

a) a produced, angular-convex clypeus without a median notch,
b) a slender, moderately bent scape base without edges or carinae,
c) absence of any angularity in frontal, dorsal and caudodorsal parts of petiole profile,

d) rather reduced sculpture and weakly developed propodeal spines.

In our opinion the three Tibetan and three Himalayan species can be combined in a *Myrmica tibetana* group which divides into a *M. tibetana* and a *M. smythiesii* complex. There are no genetic data available for the Himalayan species.

Differential diagnosis against the next similar species: The separation of *Myrmica gebaueri* sp.n. and *M. tibetana* is most difficult and a safe discrimination on the worker individual level is only possible by the multivariate analyses described above. Compared to *M. tibetana*, *M. gebaueri* sp.n. shows smaller eyes and longer and more diverging propodeal spines. A parsimonious morphometric method allows a determination on nest sample level if two or three workers per sample are measured. We simplified the species delimitation procedure by using absolute linear measurements and by reducing the number of characters for the condition that the error at nest sample level was zero. We emphasize at this point that the measuring instructions for each character have to be considered. The extracted morphometric method requires five minutes working time in a mounted specimen. With all measurements recorded in mm, a linear discriminant function

D(3) = 62.835 EYE + 44.41 SPBA - 50.213 SP - 15.713

resulted in an error of 0% on the nest sample level. Samples with D(3) < 0 are classified as *Myrmica gebaueri* sp.n., those above this threshold as *M. tibetana*. The error on the worker individual level was 5.6% in 126 specimens. The easy separation from *M. bactriana*, the three species of the *M. smythiesii* complex and from *M. tenuispina* has been treated in the section considering possible synonymies.

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