

***Apis mellifera pomonella*, a new honey bee subspecies from Central Asia**

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Abstract – Endemic honey bees of the Tien Shan Mountains in Central Asia are described as a new subspecies, *Apis mellifera pomonella*, on the basis of morphometric analyses. Principal component and discriminant analysis of the morphological characters measured clearly place these bees into the oriental evolutionary branch of honey bees, but also show that they are distinct from the other subspecies in this lineage. The existence of this newly described honey bee subspecies extends the range of endemic *A. mellifera* more than 2000 km eastward than previously estimated. Sequence analysis of mitochondrial DNA places *A. m. pomonella* within the C mitochondrial lineage (a group that is inclusive of both C and O morphological lineages). These findings support the conclusion that *A. m. pomonella* has a phylogeographic history shared with subspecies from the eastern limit of the previously known range.

Apis mellifera pomonella / new subspecies / Central Asia / taxonomy

1. INTRODUCTION

The endemic distribution of the honey bee *Apis mellifera* L. is generally considered to encompass Africa, Europe and portions of western Asia. Across this range, variation in behavior, morphology and genetic markers supports an evolutionary history of the species that includes differentiation into several major phylogenetic lineages (Ruttner, 1988; Cornuet and Garnery, 1991; Garnery et al., 1992). Based primarily on morphological characters, more than two dozen subspecies have been described within the lineages (Ruttner, 1992; Sheppard et al., 1997). These subspecies typically exhibit reduced gene flow with other such groups due to water, mountain or desert barriers and have been called “geographic races”, to reflect their adaptation to specific geographic areas (Ruttner, 1988).

Based on morphological similarities and sequence divergence among described subspecies, the speciation event that produced *Apis mellifera* has been estimated to have occurred between 0.7 to 1.3 million years ago (Ruttner, 1988; Cornuet and Garnery, 1991; Arias and Sheppard, 1996). While *Apis mellifera* occupies a large geographic distribution allopatric from the rest of the genus, a number of the other *Apis* species reside sympatrically in Asia. Western Afghanistan is considered to be the eastern limit of *A. mellifera*, with the closest proximity between *Apis mellifera* and its congeners occurring somewhere in central Afghanistan (Ruttner, 1988). Consistent with the estimated antiquity of *A. mellifera* and the paleoclimate of the region separating *A. cerana* and *A. mellifera*, Ruttner postulated that an *A. mellifera* ancestor reached western Asia about one million years ago (*ibid*).

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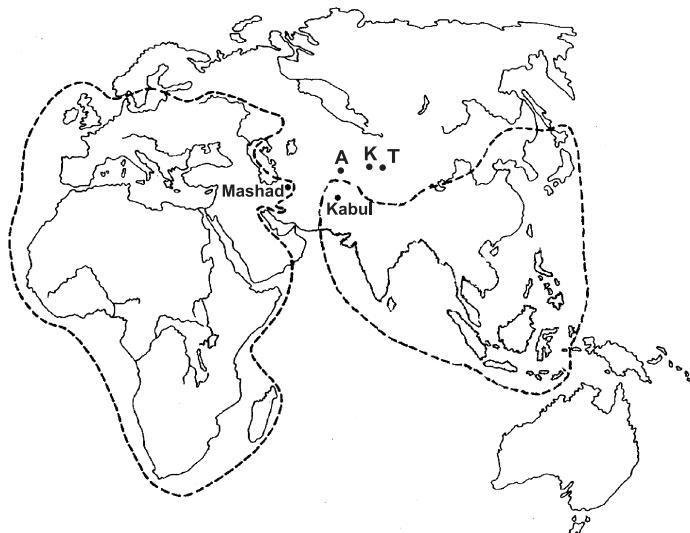


Figure 1. Areas of distribution of *Apis mellifera* and *A. cerana* (adapted from Ruttner, 1988). The collection locations in Central Asia are indicated as A (Aksu-Jabagly), K (Kurmety), and T (Tujuk).

While the relative youth of a million-year-old *A. mellifera* lineage has been supported by molecular studies, cladogenesis of *A. mellifera* and *A. cerana* appears to have occurred at a much earlier time (6–9 mya) based on allozyme and DNA sequence differences (Sheppard and Berlocher, 1989; Cornuet and Garnery, 1991; Arias et al., 1996). The apparent discrepancy between the age of *A. mellifera* subspecies and the *A. mellifera/A. cerana* cladogenesis suggests that alternative hypotheses for the origin of *A. mellifera* should be considered. These include the possibility that *A. cerana* and *A. mellifera* are not related to each other as sister taxa, rather they may be so related to as yet undescribed or extinct species. Unfortunately, perhaps the least studied area within the distribution of *Apis* is in the region between the known distribution of *Apis mellifera* and *Apis cerana*.

To further address this and other issues related to the evolution of *A. mellifera*, improved sampling of honey bee populations in regions of central Asia was undertaken. We report here the results of an investigation characterizing honey bees from the Tien Shan Mountains of Kazakhstan, extending from 1300–2000 km east of the previously known range of *Apis mellifera*.

2. MATERIALS AND METHODS

The Tien Shan Mountains lie in an east-west orientation and traverse some 1000 km from south-central Kazakhstan to western China. Adult honey bees were collected from three locations in the Tien Shan range: (1) in the western Tien Shan – two apiaries in the village of Jabagly and 5 feral colonies from the nearby Aksu-Jabagly Nature Reserve (2) in the east-central Tien Shan, an apiary near the village of Kurmetty and (3) an apiary near the village of Tujuk in the Ketman range (a branch of the eastern Tien Shan) some 100 km from the border of China and 750 km from the Jabagly collection. The sampling area in relation to the natural range of *A. mellifera* and *A. cerana* is shown in Figure 1.

Feral colonies in the Jabagly reserve were found at 2000 m and were either “caught swarms” in boxes (4) or a wild nest in a tree (1). In the Jabagly and Kurmetty apiary locations, beekeepers reported that no imported honey bees were introduced into the areas for more than 20 years. In the apiary sample taken near Tujuk, there was no recollection by local villagers that bees had ever been introduced and the apiary was composed of the descendants of wild swarms caught in previous years.

2.1. Morphometric analysis

Samples were preserved in 90% ethanol for morphometric analysis. A total of 31 samples were

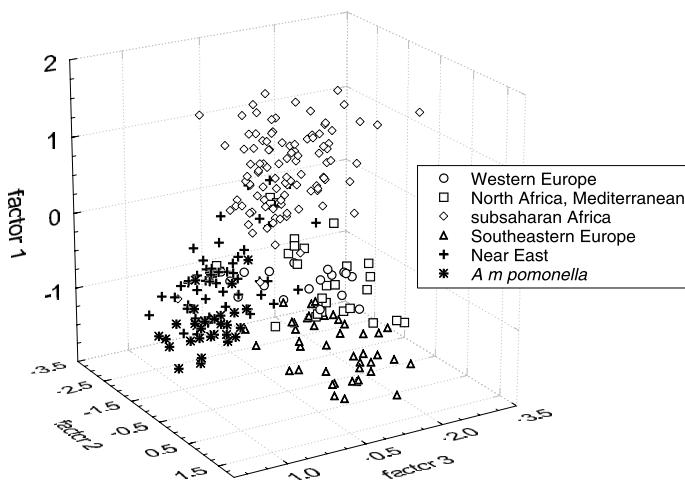


Figure 2. Positions of reference samples from all recognized subspecies of *A. mellifera* and the samples collected in the Tien Shan Mountains in a three-dimensional principal component analysis. Symbols represent individual colony samples.

subjected to morphometric analysis (15 each from Jabagly and Tujuk, 1 from Kurmetty). Fifteen worker bees per sample were dissected and measured for 39 morphometric characters according to Ruttner et al. (1978). Wing venation angles and characters of size were measured using a CCD camera combined with an on-screen measuring system (Meixner, 1994). Characters of pigmentation and pilosity were measured with a microscope and an ocular micrometer. The statistical analysis of the data was performed with SPSS for Windows and Statistica computer programs.

Multivariate statistical analyses were performed on the samples including all measured morphometric characters except width of tomentum and dark stripe on tergum 4, length of proboscis, pigmentation of tergite 2, and length of tergite 4, which were excluded due to missing data. Data of reference samples for multivariate statistics were included from the database of the Institut für Bienenkunde, Oberursel. The holotype has been deposited in the M.T. James Entomological Museum at Washington State University, Pullman, WA and paratypes have been deposited in the M.T. James Museum and in the collection at the Institut für Bienenkunde, Oberursel, Germany.

2.2. Extraction of DNA, PCR amplification and sequencing

Total DNA was extracted from five samples (two each from Aksu-Jabagly and Tujuk, and one from Kurmetty) following procedures described in Arias and Sheppard (1996). We used the polymerase chain reaction to amplify a mitochondrial DNA region that encompasses the tRNA ILE and part of the ND2 gene. The primers ILE and L1 and reaction

conditions are described in Arias and Sheppard (1996). PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

Amplification products were directly sequenced using the cycle sequencing protocol (Craxton, 1991) and an ABI 377 automated sequencer. Corresponding sequence data from other *Apis mellifera* subspecies (Arias and Sheppard, 1996) were aligned with 645 bp of amplified sequence from the Kazakhstan samples using Clustal V (Higgins and Sharp, 1988).

3. RESULTS

3.1. Morphometric analysis

To determine the position of the samples collected from the Tien Shan in the morphological tripod structure described by Ruttner (1988, 1992), an initial principal component analysis including reference samples from all subspecies of *A. mellifera* was performed. In Figure 2, the result of this analysis is presented in a three-dimensional plot. Along factor 1, mainly characters of size are included, while factors 2 and 3 contain many variables independent of size. Subspecies from Africa (diamonds) are positioned along factor 1, with subspecies belonging to the other three lineages (Ruttner, 1992) being more diverse in factors 2 and 3. The samples from the Tien Shan mountains (stars) form a compact cluster associated with the Near Eastern lineages of subspecies.

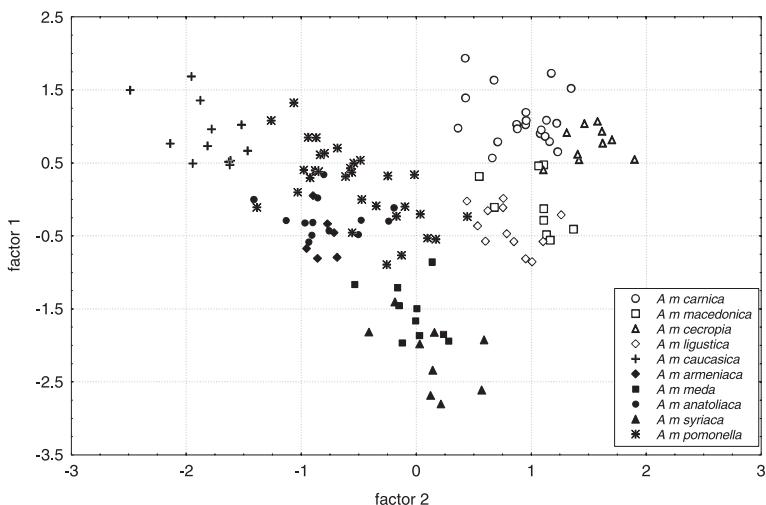


Figure 3. Position of honey bee samples in a principal component analysis performed on subspecies belonging to the C- and O-lineage (Ruttner, 1988) of *A. mellifera*. Factor 1 is plotted against factor 2. Each symbol represents one colony sample.

While this analysis provides a first impression of the homogeneity of the Tien Shan bees and their position within the oriental branch of the morphometric structure of *Apis mellifera*, it does not provide much information about the relationships of the Tien Shan bees to surrounding subspecies. However, this result demonstrates that the bees collected in the Tien Shan mountains are clearly not associated with *A. mellifera* subspecies of the M or C lineage that might have been transported into the area by migratory beekeeping practices in the former Soviet Union.

Subsequently, a principal component analysis including only the subspecies of the C- and O-branch was performed. In Figure 3, factor 1 of this analysis (accounting for 28% of the total variance) is plotted against factor 2 (20%). In this analysis, the Tien Shan bees are also clearly positioned among the lineage of the Near East, and not with the subspecies from southeast Europe.

Figure 4 shows a discriminant analysis of the same data set as in Figure 3. In Figure 4, discriminant factor 2 (24% of total variance) is plotted against factor 1 (48%). This analysis further supports the conclusion that the honey bees from the Tien Shan are part of the Oriental lineage of honey bee subspecies, without noticeable influence of commercially important subspecies from southeast Europe.

Within the Oriental branch, they form a distinctive cluster. Figure 5 shows the position of the Tien Shan samples in a discriminant analysis of the Oriental lineage. In this detailed analysis, the Tien Shan bees form a compact cluster that is distinct from all other subspecies included, mainly due to characters of wing venation in discriminant factor 2.

Based on the distinctiveness of the Tien Shan honey bee across its range from other subspecies and the similarity of the populations sampled across more than 750 km, we conclude that the Tien Shan honey bee deserves subspecific rank and propose the name, *Apis mellifera pomonella*. The type locality for *A. m. pomonella* in the Aksu river valley of Kazakhstan is shown in Figure 6.

3.2. Description of the Tien Shan honey bees

A. mellifera pomonella is a large bee, only slightly smaller than *A. m. carnica* and *A. m. caucasica*. It is in general very similar to *A. m. anatoliaca*, but has a broader abdomen and narrower tomenta. It has comparatively broad tarsi, as described to be typical for the Eastern lineage of honey bees by Ruttner (1988, 1992). Compared to its relatives *A. m. caucasica* and *A. m. armeniaca*, the proboscis of this bee is relatively short.

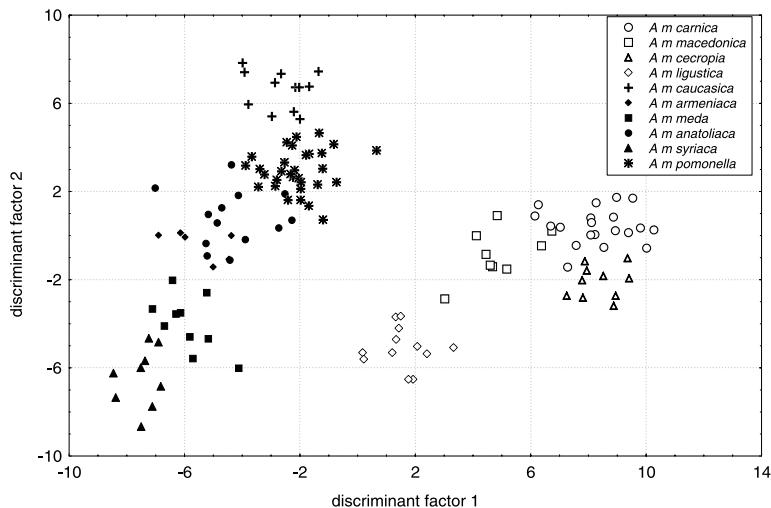


Figure 4. Positions of the same samples as in Figure 3 in a discriminant analysis. Abscissa: discriminant factor 1, ordinate: discriminant factor 2.

Although the samples were collected in altitudes up to 2000 m, the bees have comparatively short cover hair on the abdomen, reaching a mean of only 0.27 mm. Their pigmentation scores are intermediate, similar to *A. m. anatoliaca*. Mean values and standard deviations of some morphometric characters are summarized in Table I.

Beekeeping characteristics of *A. m. pomonella* appeared to be typical for a temperate mountain honey bee. Although only 25 colonies were opened for brood nest inspection during the month of June 2000, the bees were found to be quite gentle and calm on the combs during manipulation. A preliminary study of a Jabagly village apiary in June 2000 revealed that pollen forager activity (foragers returning to colonies with pollen loads) took place at a temperature threshold of 10.8 °C (Sheppard, unpublished data).

3.3. Sequence analysis of mitochondrial DNA

DNA sequences from the 5 samples were highly similar, exhibiting 0.75% sequence divergence. Sequences were deposited in Genbank under accession numbers AY135560, AY136622, AY136623, AY136624 and AY136625.

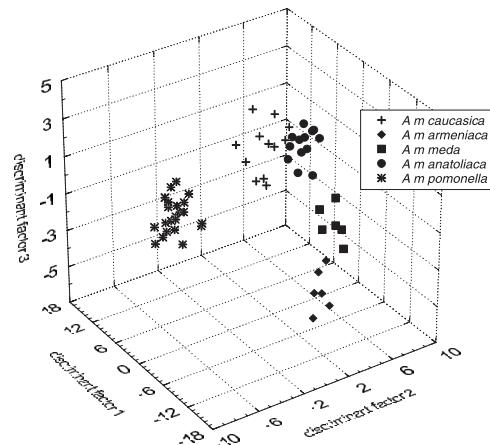


Figure 5. Positions of samples in a discriminant analysis performed on honey bee subspecies belonging to the morphological O-lineage (Ruttner, 1988) and the collection from the Tien Shan mountains.

The mean sequence divergence between the Tien Shan honey bees and other subspecies in the C lineage was less than 1.0% (*A. m. carnica*, *A. m. macedonica*, *A. m. caucasica*, range 0.16%–1.09%), while the divergences from the A-lineage and M-lineage were 1.52% (*A. m. scutellata*, range 1.10%–1.92%) and 1.65% (*A. m. mellifera*, range 1.26%–2.08%), respectively.



Figure 6. Type location for *A. m. pomonella* in the Aksu river valley, approx. 30 km southwest of the village of Jabagly, Kazakhstan. Holotype was collected from a wild colony found in a juniper tree on the slope in the left side of the photograph. Latitude 42° 20' 70 N, Longitude 70° 22' 39 E. View is from 2000 m elevation.

Inclusion of DNA sequence data from the Tien Shan bees in a phylogenetic analysis using either Parsimony or Maximum Likelihood methods did not change the overall topology of the phylogenetic tree published in Arias and Sheppard (1996). The five samples sequenced were incorporated into the C-branch subspecies group without improving the resolution within this branch (tree not shown).

4. DISCUSSION

The existence of this newly described honey bee subspecies extends the range of endemic *A. mellifera* more than 2000 km eastward than previously estimated. Further, it is likely that *A. m. pomonella* populations will be found at least to the eastern limit of the Tien Shan range in far western China (approximately 100–150 km farther east than the present Tujuk samples). Evidence for such widespread distribution of *A. m. pomonella* comes from the fact that samples from the eastern and western Tien Shan Mountains form a tightly grouped morphological cluster in our analyses. This cluster is highly distinctive and separate from European and west Asian subspecies that were brought into agricultural areas of Uzbekistan and Kazakhstan for managed beekeeping.

The finding of an endemic honey bee in this area is perhaps not surprising, given the importance of the mountainous areas of Central Asia as a center of diversity for apples,

pears and apricots. The area of the Tien Shan Mountains near Almaty (formerly known as Alma-Ata or “father of apples”), Kazakhstan, is the area of greatest genetic diversity for a wild *Malus* species, *M. sieversii*, that is the predominant ancestor of domesticated apple varieties (Hokanson et al., 1997). As a horticultural crop, apples are self-incompatible and typically pollinated by honey bees on a commercial scale. Although a careful study of the pollinating insects of the wild apple forests of Kazakhstan is lacking, the sympatric distribution of the new subspecies with wild apples throughout the sampled areas of the Tien Shan in this study provide the basis for the trinomial epithet *pomonella*, derived from the Roman deity *Pomona*, protector of gardens and fruit trees.

Sequence analysis places *A. m. pomonella* clearly within the C mitochondrial lineage (which is inclusive of both the C and O morphological lineages, sensu Ruttner, 1988), a finding consistent with the morphometric results. This supports the conclusion that *A. m. pomonella* has a phylogeographic history shared with subspecies from the eastern limit of the previously known range. However, the question is raised as to whether *A. m. pomonella* represents an eastward expansion of the morphological O lineage that likely originated somewhere in the region between Iran and the Mediterranean Coast (Ruttner, 1988; Franck et al., 2000). Alternatively, the Tien Shan Mountains and unknown points eastward may represent the area of origin and

Table I. Comparative characteristics of *A. m. mellifera*, *A. m. carnica*, *A. m. ligustica*, *A. m. caucasica*, *A. m. armeniaca*, *A. m. meda*, *A. m. anatoliaca* and *A. m. pomonella*. Values are means and standard deviations of samples, each sample representing one colony. N = number of sampled colonies. Measurements of size are in units of 1:100 mm; wing venation angles are in degrees. Characters of pigmentation: 0 = completely dark, 9 = completely bright (yellow). Measurements were taken according to Ruttner (1988).

	<i>mellifera</i> (N = 9)	<i>carnica</i> (N = 20)	<i>ligustica</i> (N = 12)	<i>caucasica</i> (N = 12)	<i>armeniaca</i> (N = 6)	<i>meda</i> (N = 10)	<i>anatoliaca</i> (N = 13)	<i>pomonella</i> (N = 31)
hairlength	43.47±4.77	29.57±3.74	27.92±2.35	33.22±1.96	32.53±1.55	27.39±3.17	28.86±2.98	26.85±1.95
proboscis	607.15±13.12 (N = 4)	640.87±7.51 (N = 18)	635.94±7.86	706.64±15.06	664.57±7.01	628.11±8.87 (N = 8)	643.62±17.64 (N = 12)	641.16±17.96
body size	463.02±9.58	453.44±6.25	436.93±7.8	456.62±7.76	449.85±7.34	432.9±3.59	444.98±7.32	449.49±14.04
cubital index	1.82±0.2	2.69±0.18	2.52±0.12	2.14±0.12	2.61±0.16	2.5±0.23	2.25±0.22	2.24±0.20
sternite 6 index	77.99±2.92	83.81±1.2	84.14±1.49	82.14±1.14	81.60±1.66	81.12±2.29	83.78±2.04 (N = 29)	78.18±3.03
tarsus index	54.86±1.64	55.31±1.37	55.24±0.68	57.81±0.75	57.17±0.82	56.34±1.66	57.36±0.92	56.78±1.35
pigmentation tergite 2	2.1±0.74 (N = 15)	1.39±0.95 (N = 15)	7.87±0.4	3.92±0.27	8.78±0.28	8.59±0.46 (N = 7)	5.17±1.18 (N = 7)	5.65±1.12
tonementum index	1.63±0.57	2.07±0.26	2.2±0.54	2.76±0.21	2.7±0.17	2.46±0.62	2.2±0.29	2.05±0.39

thus the other subspecies in the O lineage would represent westward expansion from the homeland of *A. m. pomonella*.

The taxonomic classification of honey bees below the species level reflects an interpretation of the extent of accumulated genetic differences between putative "subspecies" relative to the distribution of overall within-species variation (Hepburn and Radloff, 1997; Sheppard, 1997). As a further complication, there are examples of gene flow between described subspecies. This phenomenon has been studied and described in terms of genetic introgression and hybridization using a variety of molecular markers (Badino et al., 1984, 1988; Cornuet et al., 1986; Smith et al., 1991; Meixner et al., 1993; Hepburn et al., 1998; Franck et al., 2000). Notable examples include northeastern and northwestern Italy, where genetic introgression of *A. m. ligustica* occurs with neighboring *A. m. carnica* and *A. m. mellifera*, respectively. Evaluation of honey bee populations from these and other areas of introgression with adequate multivariate morphometric analysis or with suitable genetic markers, can discern the hybrid background. Based on the tight morphological clustering of the Tien Shan honey bee from across the sampled range and the distance from other known subspecies, we do not consider it likely that there is widespread gene flow between *A. m. pomonella* and any of the western Asian subspecies in the O lineage.

The description of this new honey bee subspecies is not directly consequential to the issue of sister taxon status between *A. mellifera* and *A. cerana*. Further studies of wild honey bee populations in other regions of central Asia, especially in areas closer to the natural range of *A. cerana*, are needed to develop an adequate understanding of this aspect of *Apis* phylogeography.

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Résumé – *Apis mellifera pomonella*, nouvelle sous-espèce de l'Abeille domestique en Asie centrale. Les abeilles domestiques endémiques des Monts Tien Shan d'Asie centrale sont décrites comme nouvelle sous-espèce, *Apis mellifera pomonella*, sur la base d'analyses multivariées des caractères morphométriques. Les analyses morphométriques ont porté sur 31 échantillons d'abeilles provenant de lieux situés dans les zones est et ouest des Monts Tien Shan (Fig. 1). Les analyses en composantes principales et les analyses discriminantes prouvent que ces échantillons ne sont pas associés aux sous-espèces d'*A. mellifera* qui auraient pu être importées dans la région par les pratiques d'apiculture transhumante dans l'ancienne Union Soviétique. Elles forment au contraire un groupe distinct qui se rattache à la branche évolutive orientale des abeilles domestiques (Figs. 2 à 5). Ces résultats étendent l'aire de répartition d'*A. mellifera* de plus de 2000 km vers l'est par rapport aux précédentes estimations. *A. m. pomonella* est une grosse abeille, seulement un peu plus petite qu'*A. m. caucasica*. Elle se caractérise par un large abdomen et de larges tarses, deux caractères typiques des sous-espèces de la lignée orientale de l'Abeille domestique. Contrairement à *A. m. caucasica*, les abeilles des Monts Tien Shan ont un proboscis plutôt court. Le tableau I donne les moyennes et les écarts-types de certains caractères morphologiques d'*A. m. pomonella*, ainsi que ceux des sous-espèces environnantes. Les données des séquences mitochondrielles confirment aussi qu'*A. m. pomonella* partage une histoire évolutive avec la lignée orientale et du sud-est de l'Europe, mais ne permettent pas de résoudre l'origine de cette sous-espèce. L'aire de répartition de cette nouvelle sous-espèce d'*A. mellifera* en Asie centrale est considérée comme étant le centre de la diversité génétique pour les pommes et d'autres espèces d'arbres fruitiers, d'où le nom proposé pour cette nouvelle sous-espèce : *Apis mellifera pomonella*.

Apis mellifera pomonella / Asie centrale / sous-espèce nouvelle / taxonomie

Zusammenfassung – *Apis mellifera pomonella*, eine neue Unterart der Honigbiene in Mittelasien. Honigbienen aus dem Tien Shan Gebirge in Zentralasien werden auf der Grundlage von multivariaten Analysen morphologischer Eigenschaften als neue Unterart *Apis mellifera pomonella* beschrieben. Insgesamt wurden 31 Bienenproben von Sammelpunkten im westlichen und östlichen Tien Shan Gebirge morphometrisch analysiert. Die Ergebnisse der Hauptkomponentenanalyse und Diskriminanzanalyse zeigten, dass diese Proben nicht mit anderen Unterarten von *A. mellifera* assoziiert sind, die durch kommerzielle Wanderimkerei in der früheren Sowjetunion in diese Gegend gebracht worden sein könnten. Statt dessen werden sie klar in die orientalische Evolutionslinie der

Honigbienen eingeordnet und erweitern damit das Verbreitungsgebiet von *Apis mellifera* um mehr als 2000 km weiter nach Osten als zuvor angenommen. *A. m. pomonella* ist eine grosse Biene, nur unwe sentlich kleiner als *A. m. caucasica*. Sie kann weiter durch ein breites Abdomen und breite Tarsen gekennzeichnet werden, beides für die orientalische Linie typische Eigenschaften. Im Gegensatz zu *A. m. caucasica* haben die Bienen des Tien Shan Gebirges jedoch einen relativ kurzen Rüssel. Mittelwerte und Standardabweichungen einiger morphologischer Charaktere von *A. m. pomonella* und benachbarter Unterarten sind in Tabelle I zusammengefasst. Sequenzdaten aus dem mitochondrialen Genom unterstützen ebenfalls die Annahme einer gemeinsamen Evolutions geschichte von *A. m. pomonella* und der südosteuropäischen und orientalischen Evolutionslinie der Honigbienen, lösen jedoch die mögliche Herkunft dieser neuen Unterart nicht weiter auf.

Das Verbreitungsgebiet der neuen Unterart von *Apis mellifera* in Zentralasien wird als Zentrum der genetischen Vielfalt für Äpfel und andere Arten von Baumfrüchten betrachtet. Als Name für die neue Unterart wird deshalb *Apis mellifera pomonella* vorgeschlagen.

Apis mellifera pomonella / Mittelasien / neue Unterart / Taxonomie

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