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Original article

Apis nulensis Tingek, Koeniger and Koeniger, 1996 and its genetic relationship with sympatric species inferred from DNA sequences

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Summary — *Apis nuluensis* Tingek, Koeniger and Koeniger is a new species recently described by Tingek et al (1996) on the basis of morphological and behavioral characters. To understand the genetic relationship between the new species and sympatric species of the *A cerana* group from Borneo, two different DNA regions of nuclear (EF-1α intron) and mitochondrial (ND2 gene) origin were amplified and sequenced. Phylogenetic analyses of the sequences indicate that *A nuluensis* and *A cerana* are sister taxa. The apparent paraphyly of *A cerana* with respect to *A nuluensis*, may result from relatively recent speciation of *A nuluensis* from the isolated *A cerana* population of Borneo. However, further studies of *A cerana* across its range are needed to clarify the status of Bornean populations.

***Apis nuluensis* / phylogeny / molecular systematics / DNA / nucleotide sequence**

INTRODUCTION

Although Maa (1953) recognized 24 different species in his revision of the genus *Apis*, until the late 1980s it was generally accepted that four species existed. Recently, two additional species from Asia were described, *Apis andreniformis* (Wu and Kuang, 1987) and *Apis koschevnikovi* (Tingek et al, 1988; Ruttner et al, 1989). The identification of *Apis* species and discussion of honey bee phylogeny has mainly been based on morphological characters, especially the male genitalia (Simpson, 1960; Ruttner, 1988; Koeniger et al, 1990; Alexander, 1991), although behavioral differences, such as variation in nest construction are often cited (Koeniger, 1976; Ruttner, 1988). This latter feature distinguishes the honey bees into two distinct groups: the open-nesting honey bees (*Apis florea, A andreniformis* and *A dorsata*) and the cavity-nesting honey bees (*Apis mellifera, Apis cerana* and *A koschevnikovi*). According to morphology,

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three distinct body size groups are found: 1. *A dorsata*; 2. *A florea-andreniformis*; and 3. *A cerana-mellifera-koschevnikovi*.

Recently Tingek et al (1996) collected a new species of *Apis* in Borneo (Indonesia), a cavity-nesting honey bee that, morphologically, belongs to the *A cerana-mellifera-koschevnikovi* group. The new species was named *Apis nuluensis*. Its distribution is restricted to mountain areas in Borneo and the bees are morphologically distinct from the sympatric species *A cerana* and *A koschevnikovi* (Tingek et al, 1996). The studies conducted on mating behavior showed that *A nuluensis* drones have a different flight time compared with the other sympatric species. In areas where the three species occur, no hybrids were found and analysis of morphological and behavioral characters well support *A nuluensis* as a valid biological species (Koeniger et al, 1996).

The development of molecular techniques in the past decades has enabled researchers to characterize species and populations with specific DNA markers or enzyme polymorphisms (Berlocher, 1984; Wilson et al, 1985; Avise, 1986; Harrison, 1989). The analysis of DNA sequences has been successful for estimating phylogenetic relationships among different subspecies and species of honey bees (Garnery et al, 1991, 1992; Willis et al, 1992; Arias and Sheppard, 1996; Sheppard and Arias, unpublished data). In this study we analyzed DNA sequences from the mitochondrial ND2 (NADH dehydrogenase) gene and from the nuclear intron EF-1a (elongation factor 1) to address genetic relationships between *A nuluensis* and other *Apis* species.

MATERIALS AND METHODS

Adult workers of the various species were collected from wild colonies and frozen or stored in EtOH for subsequent analysis (table I). We extracted total DNA from individual workers from eight colonies: *A cerana* (3), *A koschevnikovi* (2), *A mellifera* (2) and *A nuluensis* (1), according to the methods of Arias and Sheppard (1996). Two different DNA regions of mitochondrial and nuclear origin were amplified via polymerase chain reaction (PCR). A mitochondrial region of about 470 bp, encompassing part of the ND2 gene and part of the isoleucine transfer RNA gene was amplified employing the primers ILE and L2 which are described elsewhere (Arias and Sheppard, 1996). The nuclear intron EF-1α of approximately 205 bp was PCR amplified using the following primers: EF1 (5'-AAG ATC GGT GGT ATC GGT AC - 3') and EF2 (5'-TGG TGA GCG CTG CTG GAG-3'). The primers were derived from a published *A mellifera* EF-1α sequence (Walldorf and Hovermann, 1990).

PCR products were cloned using the TA Cloning Kit (Invitrogen, La Jolla, CA) and the plasmids carrying inserts were recovered by alkaline lysis miniprep protocol (Sambrook et al, 1989)

Table I. Collecting sites and species names for the samples analyzed in this study.

Samples	*Species*	*Collecting sites*
CERSABA	*Apis cerana*	Tenom – Sabah – Malaysia
CERSULA	*Apis cerana*	Jenepanto – Sulawesi – Indonesia
CERSLAN	*Apis cerana*	Sri Lanka
KOSSAB1	*Apis koschevnikovi*	Tenom – Sabah – Malaysia
KOSSAB2	*Apis koschevnikovi*	Tenom – Sabah – Malaysia
MELLIF1	*Apis mellifera*	Torino – Italy
MELLIF2	*Apis mellifera*	Assiut – Egypt
NULSABA	*Apis nuluensis*	Tenom – Sabah – Malaysia

followed by *Eco*R I digestion. The fragments were separated in 1.2% agarose gels and preparations showing the expected fragment size for ND2 and *EF*-1α were denatured (Sambrook et al, 1989) prior to the sequencing reaction. Sequencing was carried out according to Sanger methods (Sanger et al, 1977).

One or two clones per individual were completely sequenced for each region studied (ND2 and EF-1α) using M13 universal primers (M13 F 5'-TGA AAA CGA CGG CCA G-3' and M13 R 5'-CAG GAA ACA GCT ATG AC-3'). We obtained the entire sequence for each of the two regions without using internal primers.

The sequences were aligned using the multiple-sequence-alignment program CLUSTAL V (Higgins and Sharp, 1988) and, subsequently, by eye. The MEGA program version 1.01 (Kumar et al, 1993) was used to estimate evolutionary distances and nucleotide statistics. The phylogenetic analysis was performed using neighbor-joining (Saitou and Nei, 1987) and parsimony (PAUP 3.1; Swofford, 1993). Confidence probabilities for tree branches were calculated based on a standard error test (Rzhetsky and Nei, 1992). Gaps were considered as a simple event in the EF-1α alignment.

RESULTS AND DISCUSSION

Sequences were obtained from the ND2 mitochondrial gene (417 bp) and from the EF-1α intron (199–209 bp) of four different cavity-nesting *Apis* species. The sites showing base substitutions are shown in figures 1 and 2. The number of base substitutions and sequence divergence in pairwise comparisons are shown in tables II and III for the ND2 region and EF-1α, respectively.

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                                                                   111 111
                111 222 223 333 334 444 444 555 566 667 777 788 889 999 001 112
        345 679 058 245 793 467 890 235 689 124 803 673 567 824 780 479 021 240
CERSABA TTT ATC ATT CTA TTC ATG CGT ATA TAG ATA ACT GCT AAA TGA TGC TGC ATT AAT
CERSLAN ... ... ... ... ..T ... .A. ... C.A ... .TA AT. ... ... ..A ..T ... ..C
CERSULA ... ... ... ... ... ... ... .CT ... ... ... AT. ... ..C .AT ... ... ...
KOSSAB1 ..C .GA CCA ... ..T GAA TTA TCT .TA .A. GAA AAA .TG ... .AT ..T ..C ...
KOSSAB2 ..C .GA CCA ... ..T GAA TTA TCT .TA .A. GAA AAA .TG ... .AT ..T ... ...
MELLIF1 C.. C.. ... TCC CGT ..T AT. T.. ATA TAT GAA AA. C.. .AT .AT .AA TA. GTC
NULSABA .C. ..T ... ... ... ... ... .CT ... ... ..A AA. T.. C.C CAT AA. ... T..

        111 111 111 111 111 111 122 222 222 222 222 222 222 222 222 222 222 233
        223 455 667 778 888 888 900 111 112 333 344 444 455 566 677 888 899 900
        365 101 122 470 123 469 224 346 796 145 601 345 626 823 447 356 813 489
CERSABA TAA TTC TCA TTT ATT GTA AAT TTA TTA TAA GAT AGG TAT TCA ACA AGT ATT CTT
CERSLAN C.G ... .T. ... ... ..T T.. ... ... ... ... .A. ... ... .T. TA. .C. T..
CERSULA ... ... .T. C.. ... ... ... ... ... ... ..A ..A ... ... ... .A. G.. ...
KOSSAB1 ... .A. .TT .C. ..A A.. .GA ... ... .T. .T. ..A AT. ... .T. .TC ..A T.A
KOSSAB2 ... .A. .T. .C. ..A A.. .GA ... ... .T. .T. ..A AT. ... .T. .TC ..A T.A
MELLIF1 ... .CA C.. ... TCA .A. ... C.T AAG C.T CCA TA. ATA AAC TAG .AA ... TC.
NULSABA .T. C.. .T. ..C G.. ... T.. .C. ... ... ... ..A .T. ... .T. TA. ... T..

       .333 333 333 333 4
        112 333 566 679 1
        253 569 404 686 6
CERSABA TAT ACA CAA TTT C
CERSLAN ... .T. T.. CAC .
CERSULA ... .T. T.. .A. G
KOSSAB1 ..A TTT T.. .A. .
KOSSAB2 ..A TTT T.. .A. .
MELLIF1 CC. .TT TG. .A. .
NULSABA C.. .T. T.C .A. .
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Fig 1. Variable sites for the ND2 mitochondrial gene. The first substitution (number 3) corresponds to position 504 from published mitochondrial DNA sequence (Crozier and Crozier, 1993) and the other number follow the order. Dots indicate nucleotide identity with the first time (CERSABA). The complete sequences have been deposited in GenBank.

Table II. Pairwise comparisons of a fragment of 417 bp from the mitochondrial ND2 gene region.

	CERSABA	*CERSLAN*	*CERSULA*	*KOSSAB1*	*KOSSAB2*	*MELLIF1*	*NULSABA*
CERSABA		27	17	56	54	74	34
CERSLAN	6.47		29	56	54	72	37
CERSULA	4.08	6.95		51	49	73	26
KOSSAB1	13.43	13.43	12.23		2	80	58
KOSSAB2	12.95	12.95	11.75	0.48		78	56
MELLIF1	17.75	17.27	17.50	19.18	18.70		77
NULSABA	8.15	8.87	6.23	13.91	13.43	18.46	

The numbers above and below the diagonal represent the number of substitutions and the sequence divergence (%), respectively.

The neighbor-joining and parsimony phylogenetic analyses for both regions showed nearly the same tree topology (figs 3–5). Three distinct groups can be described: A) *koschevnikovi*-group which includes the two samples of this species analyzed from Sabah; B) *cerana*-group which includes all *A cerana* samples collected in three different locations (Sabah, Sri Lanka, and Sulawesi) and the new species collected in Sabah; and C) *mellifera* which was used as outgroup. The EF-1α intron showed only a few characters supporting each tree branch, mainly for groups A and B. Surprisingly, despite the few characters, neighbor-joining analysis produced a tree where *A cerana* samples were grouped separately from *A nuluensis* (fig 5), placing it as the sister taxon. In contrast, the ND2 region yielded a robust data set, maintaining the same tree topology under different phylogenetic analyses, with the clade containing *A cerana* and *A nuluensis* well supported (fig 4).

The fact that *A nuluensis* clustered together with *A cerana*, rather than as a separate group as observed for *A koschevnikovi*, suggests that *A nuluensis* diverged from *A cerana* more recently. Thus, although *A nuluensis* is morphologically distinct from *A cerana,* molecular analyses indi-

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                    1111111111 1111112
          1123557789 0001112667 7788991
          8946375821 5673671355 8929064
CERSABA   AGATATGAGC TATGCGACTG GTGGAGC
CERSLAN   .......... .......... .......
CERSULA   .......... .......... .......
KOSSAB1   ......C... .......... .....A.
KOSSAB2   ......C... ...CGC.... .....A.
MELLIF2   G.GCGCC--T AGC...GTCA ACTAG.G
NULSABA   .T.....CA. .....-.... .......
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Fig 2. Elongation factor I (EF-1α) intron variable sites. The number 18 corresponds to the nucleotide number 1201 from published EF1 sequence (Walldorf and Hovemann, 1990) and the other numbers follow the sequence. Dotes indicate nucleotide identity with the first line (CERSABA). Gaps are indicated by bars. The complete sequences have been deposited in GenBank.

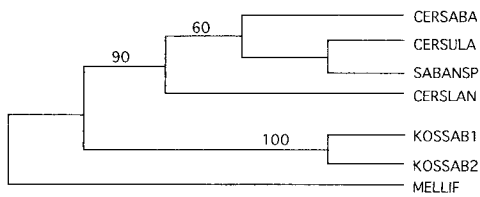

Fig 3. Parsimony tree obtained from ND2 mitochondrial gene sequence data and rooted using *A mellifera* sequence. Bootstra frequencies are shown for each node. The tree length is 156 steps, CI = 0.869 and RI = 0.712.

Table III. Pairwise comparisons of a fragment of 199-209 bp long from the EF-1α nuclear intron.

	CERSABA	*CERSLAN*	*CERSULA*	*KOSSAB1*	*KOSSAB2*	*MELLIF1*	*NULSABA*
CERSABA		0	0	2	5	20	3
CERSLAN	0.00		0	2	5	20	3
CERSULA	0.00	0.00		2	5	20	3
KOSSAB1	0.97	0.96	0.96		3	20	5
KOSSAB2	2.41	2.40	2.40	1.43		23	6
MELLIF1	10.0	9.95	9.95	9.90	11.39		21
NULSABA	1.47	1.45	1.45	2.43	2.91	10.55	

The numbers above and below the diagonal represent the number of substitutions and the sequence divergence (%), respectively.

cate that, for the nuclear and mitochondrial regions examined, few mutations have accumulated since the two species diverged. Through computer simulations, Neigel and Avise (1986) verified that recently diverged species are more likely to share mtDNA haplotypes with each other than with different lineages of the same species. We can correlate our results with Neigel and Avise's (1986) IIIa scheme of phylogenetic relationship, with *A cerana* being paraphyletic with respect to *A nuluensis.* The observation of paraphyly and polyphyly at this level can be explained by the presence of alleles before the phylogenetic divergence of species or morphological types (Neigel and Avise, 1986). Vogler and DeSalle (1993) reported a similar observation for populations of different tiger beetle subspecies, and postulated that the distribution of mtDNA haplotypes was already in place when the overlying pattern of morphology was established.

The sequence divergence within *koschevnikovi*-group for the ND2 region was very low (0.48%) compared to *cerana*-group (4.08–6.95%) (table II). This observation should be seen with caution since the *koschevnikovi* samples were collected from the same geographic region. However, Arias and Sheppard (1996) also reported a low sequence divergence (ranging from 0–2.13%) within 14 different *A mellifera* subspecies for the same mtDNA region. This

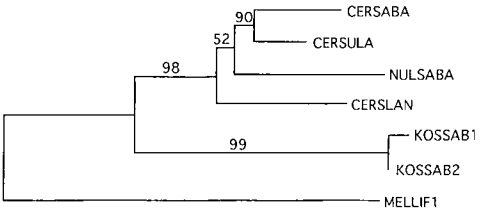

Fig 4. Neighbor-joining tree obtained from ND2 mitochondrial gene sequence. Confidence probabilities (CP) are shown on the branches. *A mellifera* sequence was used to root the tree.

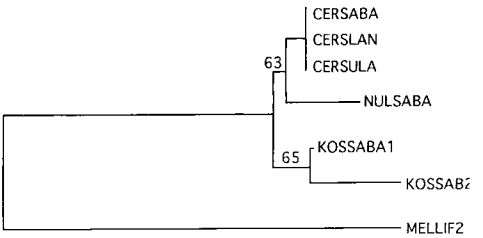

Fig 5. Neighbor-joining tree generated by EF-1α intron sequence using pairwise deletion and *A mellifera* as outgroup. Confidence probabilities (CP) are shown on the branches.

evidence suggests that the isolation of these *A cerana* populations may be relatively ancient or that our samples encompass as yet undescribed further speciation.

The molecular evidence enables us to postulate that *A nuluensis* is probably the result of recent speciation. Additional data are needed to assess the level of intraspecific variation in *A nuluensis* and to resolve other within-group relationships. However, relatively large interspecific sequence differences (eg, between *A nuluensis* and *A koschevnikovi*) are consistent with a phylogenetic analysis that places *A cerana* as the sister taxon of *A nuluensis*.

Zusammenfassung — *Apis nuluensis* Tingek, Koeniger and Koeniger, 1996 und die Ermittlung ihrer genetischen Beziehung zu sympatrischen Arten durch DNA – Sequenzanalysen. Die phylogenetische Verwandtschaft zwischen einer neu beschriebenen Honigbienenart (Tingek et al, 1996) von Sabah, Borneo und den anderen sympatrischen höhlenbrütenden Honigbienen wurde untersucht. *Apis nuluensis*, ebenfalls eine höhlenbrütende Honigbiene ist sowohl morphometrisch als auch durch ihr Verhalten von den sympatrischen Arten *Apis cerana* und *Apis koschevnikovi* getrennt (Fuchs et al, 1996; Koeniger et al 1996; Tingek et al, 1996). Molekulare Sequenzdaten wurden vom Intron EF-1α des Nukleus und den mitochondrialen ND2 – Genregionen gewonnen. Nach der Methode von Arias und Sheppard (1996) wurden von 7 Individuen alle Nucleinsäuren extrahiert: *A cerana* (3), *A koschevnikovi* (2) *Apis mellifera* (2) und *A nuluensis* (1); 4 dieser Bienenproben stammten aus Sabah (Tabelle I). Die wichtigen DNA Regionen wurden mit der Methode der Polymerase Kettenreaktion (PCR) vervielfältigt, geklont und sequenziert. Die phylogenetischen Analysen wurden mit den Methoden "Neighbor-joining" und "Parsimonia" durchgeführt. Tabelle 2 und 3 zeigen den Vergleich der Sequenzunterschiede der Bienenproben für die EF-1a bzw die ND2 Regionen. Auf der Abbildung 1 bzw 2 sind die Positionen der variablen Stellen der EF-1α bzw ND2 Regionen aufgetragen. Abbildung 3, 4 und 5 zeigen die berechneten phylogenetischen Stammbäume. Die Topologie der phylogenetischen Abzweigungen war für beide Regionen und bei den verschiedenen Methoden der phylogenetischen Analysen gleich. Zusätzlich wurden die Stammbäume aus den Sequenzdaten der EF-1α Region nur durch wenige informative Unterschiede unterstützt. *A nuluensis* wurde in allen Analysen nahe der *A cerana* Gruppe angeordnet, was ihren Status als Schwestergruppe deutlich unterstützt. Die Tatsache, daß *A nuluensis* sich zwar morphologisch von *A cerana* klar unterscheidet aber nur einige molekularen Änderungen in den beiden untersuchten Regionen aufweist, spricht dafür, daß sich seit der Trennung der beiden Arten nur wenige Mutationen angesammelt haben. Deshalb schlagen wir or *A nuluensis* als ein Ergebnis eines jüngeren Abspaltungsprozesses von der *A cerana* Gruppe vor, wobei die Kladogenese der *cerana/nuluensis* früher erfolgte als die von *cerana/koschevnikovi*.

***Apis nuluensis* / Phylogenie / molekulare Systematik / DNS / Nukleotidsequenzen**

Résumé — Les relations génétiques d'*Apis nuluensis* Tingek, Koeniger et Koeniger, 1996 avec les espèces sympatriques, déduites des données de séquence d'ADN. Les relations phylogénétiques entre l'espèce d'abeille mellifère nouvellement décrite, *Apis nuluensis* Tingek, Koeniger et Koeniger, 1996 et les autres espèces sympatriques à Sabah, Bornéo, ont été étudiées. *A nuluensis* est une abeille qui nidifie dans des cavités. Du point de vue morphologique et comportemental,

elle est distincte des espèces sympatriques *A cerana* et *A koschevnikovi* (Fuchs et al, 1996 ; Koeniger et al, 1996). Les données des séquences moléculaires ont été obtenues à partir des régions de l'intron nucléaire EF-1α et du gène mitochondrial ND2. Les acides nucléiques totaux ont été extraits selon les procédures décrites par Arias et Sheppard (1996) à partir de 8 échantillons d'abeilles comprenant quatre espèces différentes : *A cerana*, *A koschevnikovi*, *A mellifera* et *A nuluensis* (tableau I). Les régions présentant un intérêt ont été amplifiées par PCR, clonées et séquencées. Les analyses phylogénétiques ont été faites à l'aide des méthodes de *«neighbor-joining»* et de parcimonie. Les tableaux II et III comparent la divergence des séquences chez les différents échantillons d'abeilles pour les régions EF-1α et ND2, respectivement. Les figures 1 et 2 montrent la position des sites variables respectivement pour ces mêmes régions. Les figures 3, 4 et 5 présentent les arbres phylogénétiques obtenus. Les topologies des arbres sont les mêmes pour les deux régions étudiées et les diverses méthodes d'analyse phylogénétique. Néanmoins, les arbres basés sur les séquences des données de la région EF-1α reposent sur peu de caractères informatifs. Dans toutes nos analyses, *A nuluensis* s'agglomère au groupe d'espèces *cerana*, ce qui confirme grandement leur statut de taxons sœurs. Le fait qu'*A nuluensis* soit morphologiquement différente d'*A cerana* mais qu'elle présente peu de changements moléculaires dans les deux régions analysées indique qu'un petit nombre de mutations s'est accumulé depuis la divergence des deux espèces. Nous suggérons donc qu'*A nuluensis* est le résultat d'un processus récent de spéciation au sein du groupe d'espèces *cerana*, la séparation de *cerana* et *nuluensis* étant plus récente que celle de *cerana* et *koschevnikovi*.

***Apis nuluensis* / phylogenèse / taxonomie moléculaire / ADN / séquence de nucléotides**

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