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Sibling species of bean bruchids: a morphological and phylogenetic study of *Acanthoscelides obtectus* Say and *Acanthoscelides obvelatus* Bridwell

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Abstract

Acanthoscelides Schilsky is a large genus of neotropical bruchid beetles, in which most species show host plant specialization. *Acanthoscelides obtectus* and *Acanthoscelides obvelatus* are two sibling species specialized on *Phaseolus* beans, and are therefore considered pests. Up to now, the status of these two taxa has remained unclear, the few studies conducted having failed to elucidate whether these are two differentiated species or a single morphologically variable species. In addition, *A. obvelatus* has not been taken into account in the great majority of studies of bean bruchids. In this morphological and genetic study, we show that *A. obtectus* and *A. obvelatus* are two 'true' non-hybridizing species, which diverged about 22 Mya. Although the two species demonstrate only few morphological differences, we point out some diagnostic characters that enable their identification in the field. We also address a genetic method of differentiation of the two species, based on species-specific microsatellite loci. The strong morphological resemblance of these two species, despite their ancient divergence, may be the result of evolutionary stasis, which could be the consequence of stabilizing selection. Niche differentiation could enable the two species to coexist indefinitely.

Key words: Sibling species – bruchids – coleoptera – phylogenetic studies – evolutionary stasis

Introduction

The neotropical genus *Acanthoscelides* Schilsky (Coleoptera: Bruchidae) comprises about 300 species of seed-eating beetles (Johnson 1989). It is one of the most diverse bruchid genera. Its species are mostly specific to a narrow range of host plants, which are extremely diverse among *Acanthoscelides* species. Most feed on legumes. Among these are a group of three morphologically similar species specialized on beans of the genus *Phaseolus* (Johnson 1983): *Acanthoscelides argillaceus* Sharp, *Acanthoscelides obtectus* Say and *Acanthoscelides obvelatus* Bridwell. As suggested by recent phylogenetic studies (N. Alvarez et al., unpublished data), the three species constitute a monophyletic group within *Acanthoscelides*. However, *A. argillaceus* differs from the two other species in its specialization on species of the *P. lunatus* group, which exhibit high concentrations of cyanogenic compounds. In addition to this difference, adults of *A. argillaceus* are bright orange. In contrast, *A. obtectus* and *A. obvelatus* are specialized on beans of the *P. vulgaris* group, and exhibit darker colours. These two last species are morphologically similar (Fig. 1). Whereas *A. obtectus* was described in 1859, *A. obvelatus* remained cryptic until 1942. However, ecological and evolutionary studies usually confound the two species, which were regularly considered as one sole entity until the last decade. In fact, only very few morphological characters – essentially coloration – appear to differentiate the two species. Kingsolver (1968) described as main differences the colour of the pygidium, femur, and apical antennal segment, which are orange in *A. obtectus*, but brown-black in *A. obvelatus*. Furthermore, Kingsolver described the shape of antennae as a good discriminant character: longer and thinner segments for *A. obvelatus* and shorter and broader segments in *A. obtectus* (Fig. 2a). Among all the criteria, according to Kingsolver (1968), the most reliable character is only found in males and concerns the shape of lateral lobes of the aedeagus: smooth and thin in *A. obtectus*, sclerified and thick in *A. obvelatus* (Fig. 2b). Similarly reliable criteria have not been described in females.

The two species have overlapping environments and a single bean pod can be attacked by both species. The only marked ecological difference between the two species is their voltinism. Whereas *A. obtectus* is multivoltine and can reproduce as long as resources are available, *A. obvelatus* is univoltine and can only reproduce once a year. As a result of this difference, *A. obtectus* is now distributed worldwide, while *A. obvelatus* is still restricted to Mexico, Central America, and northern Colombia. The status of these two taxa thus remains quite mysterious. Are *A. obtectus* and *A. obvelatus* two distinct species (i.e. Is the speciation process completed?) or do their traits correspond to two extremes within a continuously varying species? Biémont et al. (1986), who found differentiation for isozyme alleles, were the first to suggest that these two sister taxa could be considered as two differentiated sibling species. However, under laboratory conditions, these authors were able to obtain a few cross-specific hybrids of *A. obvelatus* males with *A. obtectus* females after treatment of *A. obvelatus* with chemicals analogous to juvenile hormone. Nevertheless, hybridization success was poor: only half of the *A. obtectus* females laid eggs, and only 15% of eggs produced adults. Among these adults, several showed developmental abnormalities, such as antennal deformations. The authors concluded that introgression was thus possible between the two species, and postulated that such events could occur *in natura*. The discovery of several individuals with antennal deformations in the field was, according to them, a strong argument for natural genetic introgression between the two species. More recently, Gonzalez-Rodriguez et al. (2000) again conducted electrophoretic studies of isozymes on individuals of both species, and found that populations of *A. obtectus* and *A. obvelatus* presented different allele frequencies at different loci, but only

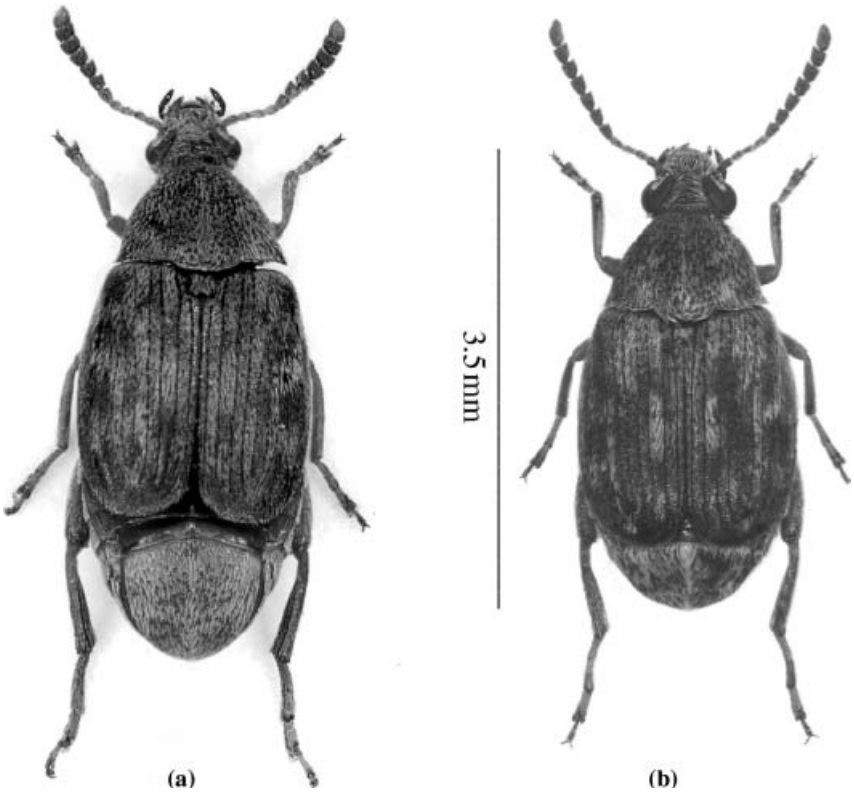

Fig. 1. Habitus (a) *A. obtectus*; (b) *A. obvelatus*. *Note*: The gap between elytra and pygidium in *A. obtectus* is artefactual and must not be taken into account

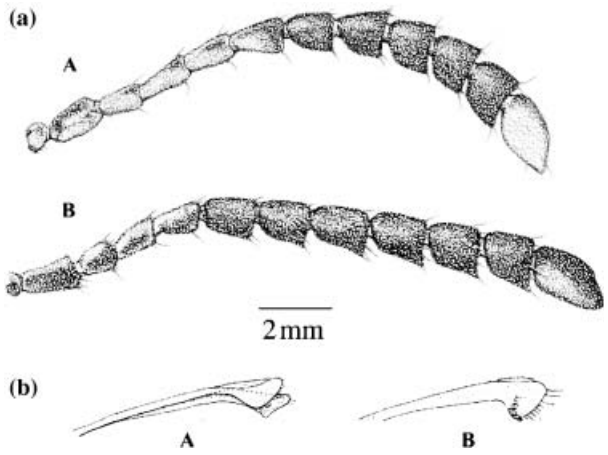

Fig. 2. (a) Antenna (A) *A. obtectus*; (B) *A. obvelatus*. (b) Lateral lobes of aedeagus. (A) *A. obtectus*; (B) *A. obvelatus*

very few private alleles. However, based on high values of Nei's genetic distance between populations of the two species, these authors concluded that the two species were differentiated, and did not interbreed *in natura*. To improve our understanding of the status of *A. obtectus* and *A. obvelatus*, we carried out morphological and DNA analyses on several populations of both species to test whether their reproductive isolation is complete. Because these beetles are economically important pests of beans, better understanding of the differences and relationships between them is required to advance research in various fields, including biological control. Indeed, most studies focused on *Acanthoscelides* associated with *Phaseolus* in Mesoamerica have up to now only considered the presence of *A. obtectus*, ignoring *A. obvelatus*. In this study, we combine morphological and genetic analysis to give a firm taxonomic footing for fundamental and applied research on these bean bruchids.

Materials and methods

Studied sites

Acanthoscelides were sampled in 21 sites between December 2001 and February 2003. Of these sites, 10 were in populations of wild beans [all sites sampled in Mexico (TLP, SJS, SAG, TEP, MAL, YAU, TLA, VDB, HUI, COP)], and 11 in populations of cultivated beans [nine sites from Mexico (SJC, SPT, YOH, OCU, SIL, STL, TZI, XOC, TEQ), one from Cameroon, and one from Switzerland] (Fig. 3 and Table 1). Emerging individuals were assigned to species on the basis of morphological characters (Kingsolver 1968).

Morphological analysis

All sampled individuals were determined using Kingsolver's criteria: (i) coloration and shape of antennae; (ii) coloration of pygidium; (iii) coloration of femur. We also analysed genitalia of all sampled males, determining each to species using this trait. Furthermore, in order to see if we could find discriminant traits in female genitalia, we dissected several females of each species, and observed the different reproductive organs.

DNA sequence analysis

We sequenced one mitochondrial gene, *COI*, and one nuclear gene, *28s rRNA*, in two individuals per species and per population. As outgroup, we used one *A. argillaceus* individual. Primer sequences were defined according to Simon et al. (1994): *C1-J-2183* and modified *TL2-N-3014* (TCCATTGCACTAATCTGCCATATTA) for *COI*; 28ee & 28 mm for *28s rRNA*. To confirm results obtained by *COI*, we sequenced another mitochondrial gene, *12S rRNA* (primers 12Sai and 12Sbi) for 10 individuals per species. Total genomic DNA was extracted using DNeasy™ kit (Qiagen, Hilden, Germany). PCR amplifications were performed in a final volume of 10 µl, which contained 1 µl of extracted DNA, 1 µl of 25 mM $MgCl_2$, 0.1 µl of

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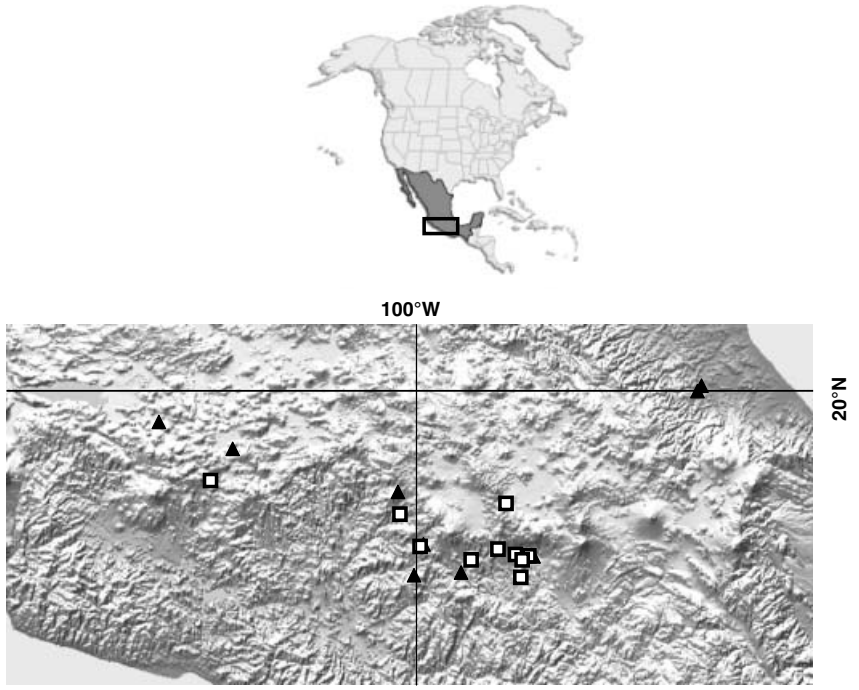

Fig. 3. Distribution of sampled populations in central Mexico. ▲, cultivated bean populations; wild bean populations

Table 1. Coordinates and altitude of all sampled populations

Abbreviations	Site name	Type of bean population	Longitude (West)	Latitude (North)	Altitude (m)
OCU	Ocumicho	c	102°13′11.8″	19°47′46.1″	2045
SIL	San Ildefonso	c	100°08′56.9″	19°22′19.8″	2400
SJC	San Jose de los Laureles pueblo	c	98°58′20.0″	18°58′40.3″	1730
SPT	San Pablo de Tejalpa	c	99°36′00.3″	18°52′59.8″	1750
STL	Santa Lucia	c	100°00′03.7″	18°52′12.5″	1790
TEQ	Tequesquipan	c	99°56′33.1″	19°03′09.2″	2300
TZI	Tzintzuntzan	c	101°34′41.5″	19°37′43.7″	1980
XOC	Xocoyolo	c	97°32′47.0″	19°58′40.0″	1550
YOH	Yohualichan	c	97°30′55.9″	20°00′56.0″	1400
COP	Copandaro	w	101°45′35.5″	19°26′24.6″	2087
HUI	Huitzilac	w	99°16′23.3″	19°01′24.4″	2544
MAL	Malinalco	w	99°30′08.9″	18°57′13.2″	1935
SAG	San Andres de los Gabeles	w	99°57′01.5″	19°02′19.5″	2280
SJS	San Jose de los Laureles campo	w	99°00′05.0″	18°58′49.7″	1855
TEP	Tepoztlan	w	99°07′15.7″	18°59′36.3″	1931
TLA	Tlayecapan	w	99°03′24.4″	18°57′20.0″	1750
TLP	Tlalpan	w	99°12′04.3″	19°17′50.3″	2403
VDB	Valle de Bravo	w	100°07′05.1″	19°13′56.8″	1918
YAU	Yautepec	w	99°01′24.0″	18°45′31.9″	1700
	Cameroon	c	Yaoundé, Province du Centre		
	Switzerland	c	Chambrelien, Canton de Neuchâtel		

c, Cultivated; w, wild.

10 mM dNTPs, 1 μl of PCR buffer (Eurogentec, Seraing, Belgium), one unit of Taq DNA polymerase (Eurogentec Red Goldstar™), 0.5 μl of forward primer, and 0.5 μl of reverse primer. PCRs were performed separately for each primer pair on a PTC-200™ thermocycler using the following cycling conditions: initial denaturation at 92°C (1 min, 30 s); 30 cycles of 92°C (30 s), 55°C (45 s), 72°C (1 min, 30 s); final elongation at 72°C (10 min). The method of Sanger (1981) was carried out using Applied Biosystems BygDye™ (Applied Biosystems, Foster City, CA, USA) protocol, and the sequences of 758 (*COI*), 384 (*12s rRNA*) and 553 (*28s rRNA*) nucleotides were obtained for each individual. Products of the sequencing reactions were then analysed on an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA). Chromatograms were manually corrected using Chromas 2.23 (Technelysium Pty Ltd, Helensvale, Australia), and sequences were aligned using ClustalW 1.83 (Thompson et al. 1994). Phylogenetic trees were reconstructed by likelihood methods using Gamma nucleotide distance models under PAUP* (Swofford 2002), according to the best phylogenetic method suggested by Modeltest 3.06 (Posada and Crandall 1998). *COI* molecular clock was tested using likelihood ratio tests, comparing likelihoods obtained by molecular clock constrained and non-constrained heuristic searches under PAUP*, using tree bisection-reconnection branch-swapping algorithm and Rambaut's parametrization of the clock. To infer a divergence time, we used a previously calibrated beetle mitochondrial clock (Gomez-Zurita et al. 2000).

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Specific analysis using DNA microsatellite loci

Using microsatellite markers developed by Alvarez et al. (2003), each extracted individual [20 per site and per species (morphologically determined)] was subjected to a PCR reaction with loci C09 (216–266 bp, specific to *A. obvelatus*) and D06 (316–366 bp, conserved locus cross-amplifying in *A. obtectus*, *A. obvelatus*, and *A. argillaceus*). Furthermore, every individual was also analysed for a third and new marker, F09 (Alvarez et al., 2004), specific to *A. obtectus* (150–170 bp). This combination of markers permitted us to assess the taxonomic status of each sampled individual of every population through one PCR reaction with three primer pairs. D06 was the *Acanthoscelides*-specific extraction control, C09 amplified only *A. obvelatus* individuals, while F09 amplified only *A. obtectus* individuals. The method theoretically permits identification of hybrids, when three distinct bands are amplified in the same individual. PCR products were revealed in 1.5% agarose gels. Non-hybrid individuals amplified two bands: D06 and C09 for *A. obvelatus* individuals, D06 and F09 for *A. obtectus* individuals. PCR amplifications were performed in a final volume of 16 µl, which contained 1.6 µl of extracted DNA, 1.04 µl of 25 mM $MgCl_2$, 1.6 µl of 10 mM dNTPs, 1.6 µl of PCR buffer (Eurogentec), 1.5 unit of Taq DNA polymerase (Eurogentec Red Goldstar^{TM}), 0.6 µl of 0.01 µM primer (for each locus, 0.6 µl forward and 0.6 µl reverse). PCRs were performed on a PTC-100^{TM} thermocycler (MJ Research, Las Vegas, NV, USA) using the following cycling conditions: initial denaturation at 92°C (1 min); seven 'touchdown' cycles: 92°C (30 s), 1°C drop per cycle to a final annealing temperature of 53°C (45 s) (Table 1), 72°C (40 s); 22 cycles of: 92°C (30 s), 53°C (45 s), 72°C (40 s); final elongation at 72°C (10 min). PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0.5X TBE buffer and 0.002% ethidium bromide. Results were displayed under ultraviolet light, using water as the negative control.

Statistical analysis

Morphological and genetic results were compared using multiple correspondence analysis under SAS v. 8.02 (SAS 1999). In the analysis, pygidium coloration, femur coloration, coloration of apical segment of antenna, and antenna shape were used as explanatory variables, while the species assignment by genetic methods (i.e. the microsatellite pattern of each individual, using primer pairs D06, C09, and F09) was treated as a supplementary variable.

Results

Morphological observations

Examination of individuals from the 20 sampled sites revealed that both *A. obtectus* and *A. obvelatus* were present in six sites (TLP, SJS, SJC, TEP, MAL, SPT), while seven (SAG, YOH, OCU, YAU, TLA, Cameroon, Switzerland) and eight (SIL, STL, TZI, VDB, HUI, XOC, TEQ, COP) sites included populations of only *A. obtectus* or only *A. obvelatus*, respectively. Two individuals from XOC and MAL presented *A. obvelatus* phenotypes, but with deformed antennae. In males, inspection of lateral lobes of the aedeagus led always to an unambiguous categorization. In females, dissections of genitalia of the two species did not detect any difference. *A. obvelatus* female genitalia are indistinguishable from those of *A. obtectus* described by Huignard (1968) (Fig. 4).

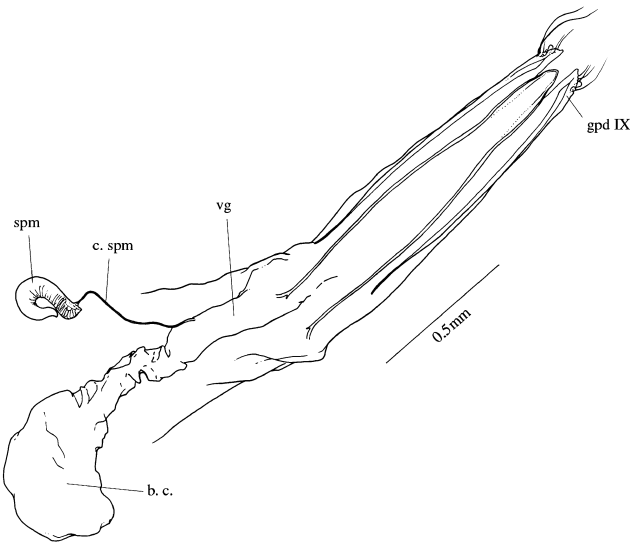

Fig. 4. Female genitalia of *A. obvelatus*. spm, spermatheca; c.spm, spermatheca canal; vg, vagina; b. c., bursa copulatrix; gpd IX, gonopode IX.

DNA sequence analysis

Topologies of phylogenetic trees were congruent for *28s rRNA* and *COI*, and showed each species as a clearly monophyletic group (Fig. 5). For *COI*, 14 and 13 haplotypes were found for *A. obvelatus* and *A. obtectus*, respectively (accession numbers AY676621–AY676675). Modeltest 3.06 suggested after a hierarchical likelihood ratio test that the best phylogenetic model was HKY85 with the following parameters: no invariant sites, gamma shape parameters = 0.2835, ts/tv ratio: 1.8576, estimated base frequencies (freqA = 0.3015; freqC = 0.1747; freqG = 0.1442; freqT = 0.3795). Within-species mean distances were 0.011 and 0.005 for *A. obvelatus* and *A. obtectus*, respectively, while the between-species mean distance was 0.167. This result was confirmed by *12S rRNA* sequences, from which two well-differentiated clades and a mean distance between the two species of 0.055 were obtained (accession numbers AY676676–AY676678; phylogenetic tree not shown). For *28S rRNA*, little genetic differentiation was found, and only two haplotypes – one for *A. obtectus* and one for *A. obvelatus* – were identified (accession numbers AY676679–AY676680). Distance (using any distance method) between the two species for this gene was 0.007. Topologies obtained for *COI*, enforcing/not enforcing molecular clock were identical for all major clades, and values for ln(likelihood) were respectively −2221.98 and −2271.76. The likelihood ratio test is marginally not passed and the molecular clock should be rejected (p = 0.031). However, as this probability is not highly significant, we will infer a molecular clock with the goal of obtaining a broad estimate of the divergence time between these two species. Following the clock developed on the beetle genus *Timarcha* (Coleoptera: Chrysomelidae) by Gomez-Zurita et al. (2000), the *COI* distance between *A. obtectus* and *A. obvelatus* corresponds to a divergence time of about 22 Mya.

Concerning the two individuals with deformed antennae (which phenotypically resembled *A. obvelatus*), their sequences showed unambiguously that they belonged to *A. obvelatus*, since the chromatogram signal was unique and because the sequence is clearly closely related to those of other *A. obvelatus* individuals.

Specific analysis using DNA microsatellite loci

Each of the 540 typed individuals (260 *A. obtectus* and 280 *A. obvelatus*) showed an unambiguous pattern, always with

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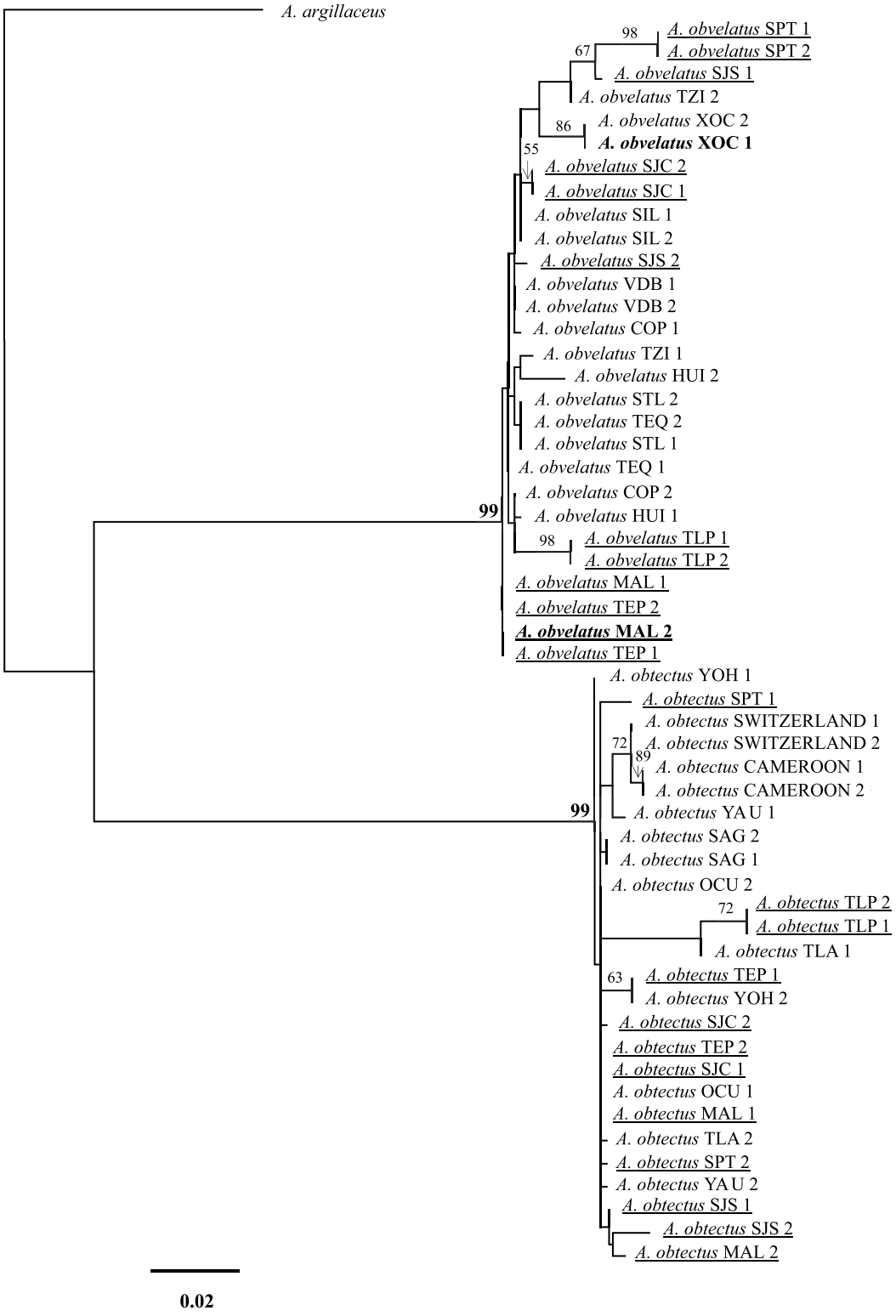

Fig. 5. Phylogenetic tree of 26 *A. obtectus* and 28 *A. obvelatus* individuals for the *COI* gene, reconstructed by maximum likelihood method. *A. argillaceus* is used as outgroup. *A. obvelatus* samples in bold correspond to individuals with deformed antennae. Underlined samples correspond to individuals from bean populations where both *A. obtectus* and *A. obvelatus* were present

two bands, either D06 and F09 for *A. obtectus* individuals, or D06 and C09 for *A. obvelatus* individuals. We found no potential hybrid, that would have borne three bands. Again, the two individuals with deformed antennae were unambiguously *A. obvelatus* individuals. Concerning the third closely related species *A. argillaceus*, only one band (for D06) was amplified. Bruchids other than *Acanthoscelides*, such as *Zabrotes subfasciatus*, seem unable to amplify any of the

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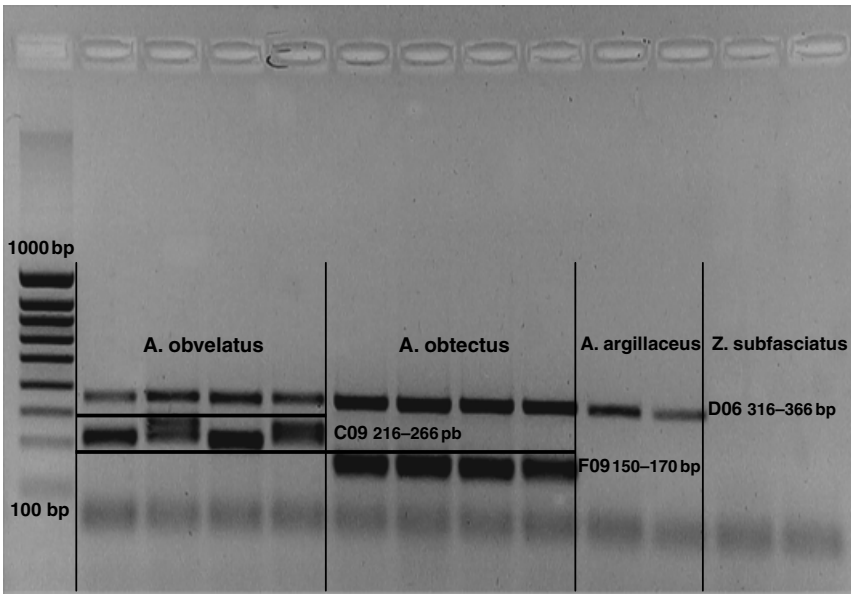

Fig. 6. Molecular diagnostic test with microsatellite loci C09, D06 and F09: *A. obvelatus* individuals amplify D06 and C09, *A. obtectus* individuals amplify D06 and F09, *A. argillaceus* individuals amplify D06 only. Individuals from other bruchid genera (e.g. *Zabrotes subfasciatus*) seem not to amplify any of the loci

specific *Acanthoscelides* microsatellite loci. Due to the fact that the three loci D06, C09, and F09 exhibit different non-overlapping ranges, the diagnostic differences are very reliable, allowing easy and unequivocal genetic determination (Fig. 6). All the results obtained with this diagnostic method were congruent with sequences revealed by *COI*, *12S*, and *28S*.

Statistical analysis

The colour and shape of antennae were by far the best morphological criteria – with the exception of male genitalia – to distinguish between *A. obvelatus* and *A. obtectus* (Fig. 7). However, in one *A. obvelatus* and four *A. obtectus* individuals the morphological (excepting genitalia in males) and genetic

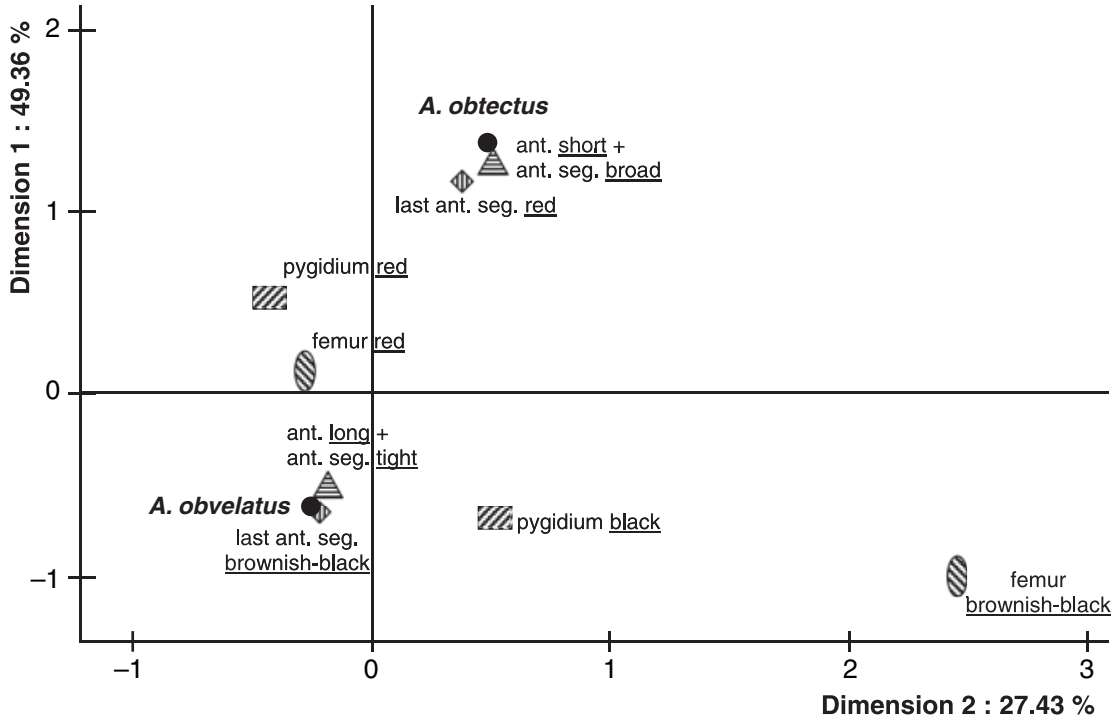

Fig. 7. Multiple correspondence analysis of morphological traits, using the species assignment by genetic methods (●) as a supplementary variable. Explanatory variables are as follows: ◬, shape of antenna; ◈, colour of last antennal segment; ▨, colour of pygidium; ▨, colour of femur

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diagnoses were not congruent. Thus, fewer than 1% of individuals (five individuals among 540 typed) could not be reliably diagnosed by morphological traits other than genitalia. In contrast, pygidium and femur colour did not allow reliable diagnosis of the species. In males, the genitalia criterion was diagnostic in 100% of cases. However, in females (in which no differences were found in genitalia), the shape and colour of the last antennal segment was the only diagnostic descriptor.

Discussion

Ancient divergence between *A. obtectus* and *A. obvelatus*

As shown by our DNA analysis, hybridization between *A. obtectus* and *A. obvelatus* does not seem to occur *in natura*. The high estimated divergence time between *A. obtectus* and *A. obvelatus* (about 22 Mya) appears to attest to a strong reproductive barrier, despite great morphological similarity. However, the use of a molecular clock calibrated for another group of species – even if that group (*Timarcha*, Chrysomelidae) is relatively closely related to Bruchidae – must be done with prudence, since the biogeographic events used by Gomez-Zurita et al. (2000) to calibrate the *Timarcha* clock are not common to *Acanthoscelides*. Furthermore, in light of the current debate on the application of molecular clocks to supposed isochronous sequences (Drummond et al. 2003), it is not easy to predict, for example, how differences in voltinism will affect the rate of neutral substitution. Nevertheless, it seems obvious that the lineages of the two species have diverged at least several million years ago. Despite the fact that the two species share exactly the same habitats in many areas of Mesoamerica, they have apparently remained two distinct non-hybridizing entities. Since none of the 54 sequenced individuals, or of the 540 genotyped individuals, yielded any indication of having issued from an interspecific cross, it is likely that the few *in vitro* hybrids obtained by Biémont et al. (1986) were the result of experimental conditions (e.g. artificially-induced hormonal manipulation), that could have modified pre- and post-zygotic barriers. Our genetic data indicate that the presence of individuals with deformed antennae *in natura* – which these authors regarded as an indication of the existence of naturally cross-specific hybrids – does not seem to be necessarily related to any introgression event.

Morphological similarity

Of all the traits studied, only the morphology of the lateral lobes of the aedeagus in males was strictly diagnostic. Female genitalia traits yielded no discriminating trait. Antennal traits (shape and colour) were also very useful and gave the right diagnosis in more than 99% of the analysed individuals, both in males and females. Other criteria, such as colours of the pygidium and femur, were not reliably diagnostic. Taking into account the ancient age of the two species – which diverged about 22 Mya – as well as their inability to interbreed, a less great morphological resemblance between *A. obtectus* and *A. obvelatus* was expected. Morphological similarity seems to be common in insects, particularly in species that diverged recently (e.g. Sharpe et al. 2000). However, some studies on arthropods have shown relatively long divergence times for pairs of sibling species, such as 5 Mya in Diplopoda (Bond and Sierwald 2002). In the last 5 years, studies have identified several groups of cryptic species in insects. To our knowledge, most studies have found congruence between the « genetic » species and their associated morphology (e.g. Sharpe et al. 2000; Sebastiani et al. 2001; Kerdelhué et al. 2002).

In the case of these two *Acanthoscelides* species, the divergence between *A. obtectus* and *A. obvelatus* seems to be more ancient than in these previous examples of sibling species of insects (presuming the applicability of the *Timarcha* molecular clock to *Acanthoscelides* species). We can postulate that, in the light of the ancient divergence time between the two species, such morphological similarity could be the consequence of evolutionary stasis, perhaps because of stabilizing selection and the negligible effect of drift. Such stasis has been reported in several species groups of vertebrates, such as stickleback fishes (Schluter and McPhail 1993) or rainforest lizards (e.g. Schneider et al. 1999), for which stabilizing selection has been proved to produce long-term morphological stability, despite very ancient divergence times. In the case of *A. obtectus* and *A. obvelatus*, a relative stability in developmental conditions of larvae in bean seeds (e.g. constraints due to seed size and to secondary chemical compounds), as well as high densities of host plant populations, may have allowed such stabilizing selection to occur. Furthermore, since metapopulations of bean bruchids seem to be fairly stable (i.e. constant large population sizes and frequent migrations between populations), drift may not have had a very strong effect as an evolutionary pressure in populations of these two species. This could explain why *A. obvelatus* and *A. obtectus* show mostly symplesiomorphic characters, despite their relatively ancient divergence. In a recent study (N. Alvarez et al., unpublished data), we showed that *A. obtectus* and *A. obvelatus* probably speciated in allopatry. According to a scenario consistent with biogeography, *A. obvelatus* originated in Mesoamerica on seasonally fruiting wild beans, where univoltinism and diapause abilities were adaptive, whereas *A. obtectus* originated in Andean South America, where it evolved adaptations to non-seasonal wild bean populations, by losing its ability to diapause and evolving multivoltinism. However, both species – even if demonstrating several ecological differences – develop on beans of the *Phaseolus vulgaris* group (common beans), whose seeds and pods are marbled and dark. Thus, the two species, which probably have evolved under similar pressures of predation and parasitism, would have benefited from traits reducing the ability of predators to detect them, and have therefore evolved similar dark colorations, mimetic with bean seeds and pods. However, since at early and intermediate stages of maturity – when females usually start laying eggs – pods are not dark enough to render beetles cryptic, the significance of their similarity in colour is difficult to evaluate. Evolutionary stasis can be invoked again, to explain the global similarity – not only in colour traits – of the two species. Indeed, examination of Johnson's keys to *Acanthoscelides* species (Johnson 1983, 1990) shows that morphological similarity of closely related species is quite common in the genus; the case of *A. obtectus* and *A. obvelatus* is not unusual.

In the same previous study (N. Alvarez et al., unpublished data), we also showed that *A. obtectus* probably reached Mesoamerica posterior to bean domestication (~7000 years ago), through human-mediated migrations. Consequently, the two sister species became sympatric only recently, compared with their time of divergence. Strong pre-zygotic and post-

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zygotic barriers have therefore probably appeared over millions of years, and prevent today any genetic exchange between the two species.

The ecological differences that may permit coexistence of *A. obtectus* and *A. obvelatus* are of great interest. Although the necessity of differentiated niches for durable coexistence is still questioned (Chesson 2000; Hubbell 2001), ecological divergence should at least facilitate coexistence. Both *A. obtectus* and *A. obvelatus* are able to develop both on wild and cultivated common beans. However, the univoltinism of *A. obvelatus* makes it more adapted to the phenology of wild beans, whereas the multivoltinism of *A. obtectus* is one of the main causes of its current cosmopolitan distribution, and of the ability of the species to develop exponentially in granaries (N. Alvarez et al., unpublished data). Nevertheless, the ecological segregation of the two species is not absolute. Several wild bean populations harboured *A. obtectus* only, whereas in some cultivated bean populations, *A. obvelatus* was strongly predominant. The presence of one or another species in a certain geographical zone is nonetheless strongly correlated with the proportion of wild versus cultivated common beans. Predicting whether one or the other *Acanthoscelides* species might be globally more competitive in Mesoamerica, perhaps even replacing the other species, is as yet impossible. Because wild and cultivated bean populations are both common in Mesoamerica, the two species could coexist indefinitely. The fact that these two morphologically similar non-hybridizing species can persist in the same habitats is most likely explained by the coexistence of wild and cultivated beans, allowing niche differentiation of the two species.

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Zusammenfassung

Schwester Arten der Bohnen Bruchiden: eine morphologische und phylogenetische Studie über Acanthoscelides obtectus und A. obvelatus

Die Acanthosceliden (*Acanthoscelides* Schilsky) sind eine grosse neotropische Gattung innerhalb der Bruchiden (Bruchidae, Coleoptera), von denen die meisten Arten eine hohe Wirtsspezifität aufweisen. *Acanthoscelides obtectus* und *A. obvelatus* sind zwei nahe verwandte Taxa, die auf *Phaseolus* Bohnen spezialisiert sind und deshalb als Schädlinge eingestuft werden. Der taxonomische Status dieser beiden Arten ist nach wie vor unklar; die wenigen Untersuchungen, die durchgeführt wurden, konnten nicht klären, ob es sich um getrennte Arten oder nur um eine, morphologisch variable Art handelt. Ausserdem wurde *A. obvelatus* in den meisten Arbeiten über Bohnen-Bruchiden nicht berücksichtigt. In der vorliegenden morphologischen und genetischen Studie zeigen wir, dass *A. obtectus* und *A. obvelatus* zwei nicht hybridisierende Taxa sind, die sich vor ca. 22 Millionen Jahren aufgespalten haben. Trotz der geringen morphologischen Unterschiede dieser beider Arten) zeigen wir einige diagnostische Parameter, die eine Identifikation im Gelände ermöglichen. Zusätzlich beschreiben wir eine genetische Methode zur Unterscheidung der beiden Arten, die auf artspezifischen Mikrosatelliten-Loci beruht. Die hohe morphologische Ähnlichkeit, die diese beiden Arten trotz der langen Zeit seit der Aufspaltung aufweisen, kann auf eine evolutionäre Stasis in Folge einer stabilisierenden Selektion zurückgeführt werden. Nischendifferenzierung könnte zu einer unbegrenzten Koexistenz dieser Arten führen.

References

Alvarez, N.; Aebi, A.; Risterucci, A. M.; Hossaert-Mckey, M.; Benrey, B., 2003: Isolation and characterization of polymorphic microsatellite loci in *Acanthoscelides obvelatus* Bridwell (Coleoptera: Bruchidae). Mol. Ecol. Notes **3,** 12–14.

Alvarez, N.; Born, C.; Risterucci, A. M.; Sourrouille, P.; Benrey, B.; Hossaert-Mckey, M., 2004: Isolation and characterization of polymorphic microsatellite loci in *Acanthoscelides obtectus* say (Coleoptera: Bruchidae). Mol. Ecol. Notes (in press).

Biémont, J. C.; Huignard, J.; Perriquet, G.; Leroi, B.; Garaud, P., 1986: L'extension de l'aire d' *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). Comparaison des populations, relations avec *Acanthoscelides obvelatus* Bridwell et analyse des processus adaptatifs. In: CNRS (ed), Lyon: Colloque National CNRS "Biologie des Populations", pp. 149–156.

Bond, J. E.; Sierwald, P., 2002: Cryptic speciation in the *Anadenobolus excisus* (Millipede) species complex on the island of Jamaica. Evolution **56,** 1123–1135.

Chesson, P., 2000: General theory of competitive coexistence in spatially varying environments. Theor. Popul. Biol. **58,** 211–237.

Drummond, A. J.; Pybus, O. G.; Rambaut, A.; Forsberg, R.; Rodrigo, A. G., 2003: Measurably evolving populations. Trends Ecol. Evol. **18,** 481–488.

Gomez-Zurita, J.; Juan, C.; Petitpierre, E., 2000: The evolutionary history of the genus *Timarcha* (Coleoptera, Chrysomelidae) inferred from mitochondrial COII gene and partial 16S rDNA sequences. Mol. Phyl. Evol. **14,** 304–317.

Gonzalez-Rodriguez, A.; Benrey, B.; Castaneda, A.; Oyama, K., 2000: Population genetic structure of *Acanthoscelides obtectus* and *A. obvelatus* (Coleoptera:Bruchidae) from wild and cultivated *Phaseolus* spp. (Leguminosae). Ann. Entomol. Soc. Am. **93,** 1100–1107.

Hubbell, S. P., 2001: The Unified Neutral Theory of Biodiversity and Biogeography. Princeton, NJ: Princeton University Press.

Huignard, J., 1968: Organisation et fonctionnement de l'appareil génital de la bruche du haricot (*Acanthoscelides obtectus*, Coléoptère, *Bruchidae*). Bulletin Biologique **2,** 233–248.

Johnson, C. D., 1983: Ecosystematics of *Acanthoscelides* (Coleoptera: Bruchidae) of southern Mexico and Central America. Misc. Publ. Entomol. Soc. Am. **56,** 1–370.

Johnson, C. D., 1989: Adaptive radiation of *Acanthoscelides* in seeds: examples of legume-bruchid interactions. In: Stirton, C. H.; Zarucchi, J. L. (eds), Advances in Legume Biology. St Louis, MO: Monographs of the Systematic Botany from the Missouri Botanical Garden, pp. 29**,** 747–779.

Johnson, C. D., 1990: Systematics of the seed beetle genus *Acanthoscelides* (Bruchidae) of northern South America. Trans. Am. Entomol Soc. **116,** 297–618.

Kerdelhué, C.; Roux-Morabito, G.; Forichon, J.; Chambon, J. -M.; Robert, A. L.; Lieutier, F., 2002: Population genetic structure of *Tomicus piniperda* L. (Curculionidae: Scolytinae) on different pine species and validation of *T. destruens* (Woll.). Mol. Ecol. **11,** 483–494.

Kingsolver, J. M., 1968: A review of the *obtectus* group in *Acanthoscelides* Shilsky, with designations of lectotypes. Proc. Entomol. Soc. Wash. **70,** 4–9.

Posada, D.; Crandall, K. A., 1998: Modeltest: testing the model of DNA substitution. Bioinformatics **14,** 817–818.

Sanger, F., 1981: Determination of nucleotide-sequences in DNA. Science **214,** 1205–1210.

SAS, 1999: Release 8.02. Cary, NC: SAS Institute.

Schluter, D.; McPhail, J. D., 1993: Character displacement and replicate adaptive radiation. Trends Ecol. Evol. **8,** 197–200.

Schneider, C. J.; Smith, T. B.; Larison, B., Moritz, C., 1999: A test of alternative models of diversification in tropical rainforests:ecological gradients vs. rainforest refugie. Proc. Natl Acad. Sci. U S A **96,** 13869–13873.

Sebastiani, F.; Meiswinkel, R.; Gomulski, L. M.; Guglielmino, C. R.; Mellor, P. S.; Malacrida, A. R.; Gasperi, G., 2001: Molecular

 doi: 10.1111/j.1439-0469.2004.00286.x

differentiation of the Old World *Culicoides imicola* species complex (Diptera, Ceratopogonidae), inferred using random amplified polymorphic DNA markers. Mol. Ecol. **10,** 1773–1786.

Sharpe, R. G.; Harbach, R. E.; Butlin, R. K., 2000: Molecular variation and phylogeny of members of the *Minimus* group of *Anopheles* subgenus *Cellia* (Diptera: Culicidae). Syst. Entomol. **25,** 263–272.

Simon, C.; Frati, F.; Beckenbach, A.; Crespi, B.; Liu, H.; Flook, P., 1994: Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. **87,** 651–701.

Swofford, D. L., 2002: PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), version 4. Sunderland, MA: Sinauer Associates.

Thompson J. D.; Higgins, D. G.; Gibson, T. J., 1994: CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. **22,** 4673–4680.

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