PERIODICUM BIOLOGORUM
VOL. 110, No 4, 329–334, 2008

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Novel morphological and genetic markers for the discrimination of three European *Pityokteines* (Coleoptera: Curculionidae: Scolytinae) species

MILAN PERNEK[1]
DIMITRIOS N. AVTZIS[2]
BORIS HRAŠOVEC[3]
DANKO DIMINIC[3]
RUDOLF WEGENSTEINER[2]
CHRISTIAN STAUFFER[2]
ANTONY I. COGNATO [5]

[1]Forest Research Institute, Jastrebarsko
Cvjetno naselje 41
10450 Jastrebarsko
Croatia

[2]Institute of Forest Entomology,
Forest Pathology and Forest Protection
Department of Forest and Soil Sciences
Boku, University of Natural Resources
and Applied Life Sciences, 1190 Vienna
Austria

[3]Faculty of Forestry, University of Zagreb
Svetošimunska 25
10000 Zagreb
Croatia

[4]Department of Entomology
Michigan State University
East Lansing, MI 48824
U.S.A.

Correspodence:
Milan Pernek
Forest Research Institute, Jastrebarsko
Cvjetno naselje 41
10450 Jastrebarsko
Croatia
E-mail: milanp@sumins.hr

Key words: Bark beetles, silver fir, morphology, mtDNA

Received September 24, 2007.

Abstract

Background and Purpose: *The three palearctic species* Pityokteines spinidens, P. curvidens *and* P. vorontzowi *are main pests on* Abies *species and their impact on Abies stands is increasing. As the three scolytid species, particularly females, are difficult to distinguish, this study aimed to find additional morphological characters for identification. Further, part of the mitochondrial COI gene was sequenced to develop a significant barcode marker for future use.*

Material and Methods: *All three bark beetle species were collected from logs in Croatia (Litorić and Trakošćan), in order to quantify the number of strial and interstrial punctures. Insect DNA was extracted and PCR products were purified, directly sequenced, aligned and analyzed by MP analysis and Bayesian analysis.*

Results and Conclusion: *The number of punctures in the first and second interstriae between the elytral base and the sutural tubercle proved to be a valuable tool for the differentiation of* P. spinidens *from* P. curvidens *and* P. vorontzowi. *This morphological feature was consistent with the number of punctures which varied for the first and the second interstriae in* P. spinidens *compared to* P. curvidens *and* P. vorontzowi. *The mitochondrial COI gene provided another means in the discrimination of* Pityokteines *species, revealing that* P. curvidens *and* P. vorontzowi *are sister species.*

INTRODUCTION

The genus *Pityokteines* (Fuchs) occurs worldwide and includes ten species: *P. elegans* (Swaine), *P. lasiocarpi* (Swaine), *P. minutus* (Swaine), *P. mystacinus* (Wood), *P. ornatus* (Swaine), *P. sparsus* (LeConte), *P. curvidens* (Germar), *P. spinidens* (Reitter), *P. vorontzowi* (Jacobson) and *P. marketae* (Knizek). The former six species occur in Nearctic while the latter four occur in the Palearctic (*1, 2*). *P. marketae* was described in Turkey and no further finding was reported since then (*2*). *P. spinidens* and *P. vorontzowi* occur in *Abies* habitat from the Pyrenees to the Caucasus Mountain and *P. curvidens* additionally in Asia minor and Japan according to Pfeffer (*3*). All species are phloeophagous and polygynous, spending most of their life cycle under the bark besides a short dispersal period as adults. The pupal stage occurs inside the sapwood (*4*). *P. curvidens* and *P. spinidens* usually breed in the lower trunk of silver fir trees (*Abies alba* Mill.), while *P. vorontzowi* usually occupies the upper

crown *(5)*. The three palearctic *Pityokteines* species often show relatively high levels of aggressiveness and thus are considered to be economically important forest pests. They have been reported as an important factor in silver fir decline in some parts of Europe, a complex disease caused by a variety of abiotic and biotic factors (*6*). *P. curvidens*, *P. spinidens* and *P. vorontzowi* are commonly found in Croatia and have been associated with increased levels of silver fir mortality since the beginning of 2000 (*7*).

During mass infestations these species have contributed greatly to the damage of silver fir in the southern parts of Europe (*7–9*). In Croatia, silver fir belongs to the economically important tree species and outbreaks of the fir bark beetles resulted in timber loss of a few thousand m^3 in 2002, about 100.000 m^3 in 2003, about 130.000 m^3 in 2004 and more than 300.000 m^3 in 2005 (*7*). Most probably, drought increased the attractiveness of declining trees to beetles whereas high temperatures favored beetles' development (*7*).

Increased research aiming to control these beetles requires a rapid and accurate means of species identification. The existing taxonomic keys for the palearctic group of *Pityokteines* (*3, 10, 11*) caused difficulties as the average body size of two species is greater for *P. curvidens* and *P. spinidens* compared with *P. vorontzowi* (*3, 12, 13*). Furthermore, the described characters like length of the setae on the anterior margin of the pronotum of females or the position of the first sutural tubercle of males are variable and often lack accuracy.

Recent advances in molecular techniques have assisted in the development of another valuable tool in the resolution of taxonomical questions. DNA barcoding involves the sequencing of a signature region of the mitochondrial genome in order to identify inter- or intraspecific differences (*14, 15*). Among 13 mitochondrial DNA (mtDNA) protein coding genes, Cytochrome Oxidase subunit one (COI) demonstrated a slow rate of amino acid change (*16*).

This study aims towards description of novel morphological characters and defining the three species by a partial region of the mitochondrial COI gene to facilitate fast identification of the three species by non-taxonomists.

MATERIAL AND METHODS

Morphological identification

Light microscopy

All three bark beetle species were collected from logs in Litorić (550 m) and Trakošćan (400 m), two natural fir forests in Croatia (Table 1). At least 52 specimens were randomly selected for each of the three *Pityokteines* species in order to quantify the number of strial and interstrial punctures (Table 2). Several other morphological

TABLE 1

Information on the locations, both situated in Croatia.

	Location	n_m	n_g	Alt.(m)	Long.	Lat.
P. curvidens	Litorić	8	1	550	15°04'E	45°27'N
	Trakoščan	9	–	450	15°56'E	46°05'N
P. spinidens	Litorić	10	1	550	15°04'E	45°27'N
	Trakoščan	7	–	400	15°56'E	46°05'N
P. vorontzowi	Litorić	7	1	600	15°04'E	45°27'N
	Trakoščan	8	–	450	15°56'E	46°05'N
	Total	49	3			

TABLE 2

The number of punctures in the first (1s) and second stria (2s) and first (1i) and second interstria (2i) between the elytra base and sutural tubercle of *P. curvidens*, *P. spinidens* and *P. vorontzowi*.

		Number of individuals analyzed	*P. curvidens*	*P. spinidens*	*P. vorontzowi*
1i	♂	26	10.2 ± 1.03	5.9 ± 1.14	9.4 ± 0.93
	♀	26	10.2 ± 1.15	6.0 ± 1.03	9.5 ± 1.04
2i	♂	26	10.7 ± 1.23	6.2 ± 1.23	9.6 ± 1.12
	♀	26	10.6 ± 1.43	6.8 ± 1.30	10.3 ± 1.01
1s	♂	26	12.1 ± 1.10	13.2 ± 1.04	10.4 ± 1.12
	♀	26	14.6 ± 1.85	15.2 ± 1.70	13.1 ± 1.26
2s	♂	26	12.1 ± 1.27	13.5 ± 1.34	10.8 ± 1.38
	♀	26	14.6 ± 1.48	15.5 ± 1.53	13.3 ± 1.43

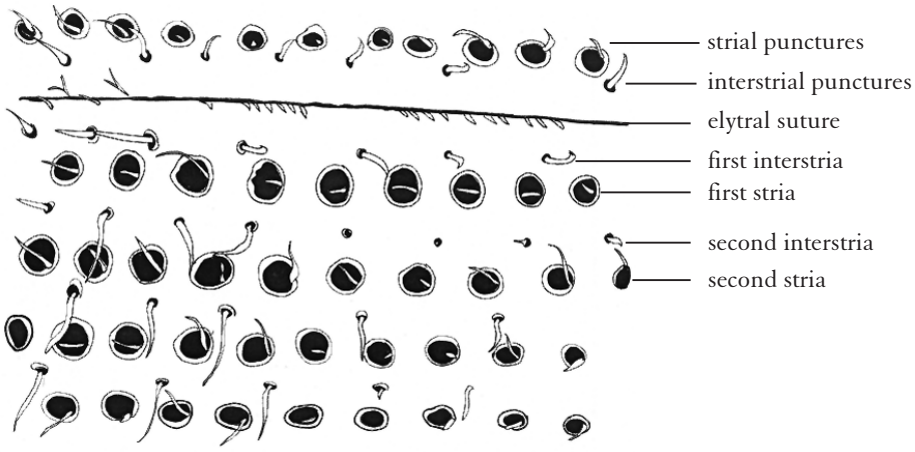

Figure 1. *Details of strial punctures and punctures on interstriae on the elytra of P. curvidens.*

features, such as antennae and cuticular structures, were investigated for variation. The strial and interstrial punctures (Figure 1) were counted in the first and second elytral stria and interstria beginning from the elytral base to the sutural tubercle (at the base of the elytral declivity) under a stereo microscope at magnification of 40x. The numerical data were analyzed using StatSoft, Inc. (2005), STATISTICA (data analysis software system), version 7.1.

Scanning electron microscopy

For the purpose of taking scanning electron microphotographs, 49 adult beetles were collected from logs in Litorić and Trakošćan (Table 1). The beetles were washed in acetone using an ultrasonic bath (Bandelin, Sonorex TK 20 R). After drying, the beetles were mounted on specimen stubs directly with conductive silver. The specimens were sputtered in a SEM coating system with gold palladium using a voltage of 2 kV and current intensity of 20 mA for 90 under vacuum of 0.2–10 mbar (Polaron E5100, Hatfield, PA, USA). Electron micrographs were taken with the JEOL JSM 5200 scanning microscope (JEOL, Tokyo, Japan).

Molecular identification

The three species were collected in Litorić (Table 1) and stored in absolute ethanol at –20 °C. Insect DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, USA) following the manufacturer's protocol and eluted in 50 µl elution buffer. Amplification was carried out in 50 µl reactions containing 3.75 µM $MgCl_2$ 125 µM dNTPs (Fermentas, Lithuania), 0.5 µM of forward primer UEA5 and 0.5 µM of reverse primer UEA10 (*17*) and 1U of Biotherm Taq (Genecraft, Germany). Thermocycling was performed in a Primus 25 advanced Thermocycler (PeqLab, Germany) and consisted of an initial denaturation step of 3 min at 94 °C, which was followed by 33 cycles at 94 °C (30 s), 48 °C (60 s) and 72 °C (90 s) and a final extension step at 72 °C (10 min). PCR products were purified using the QIAquick™ PCR Purification Kit (QiaGen, Austria) and directly sequenced with UEA10 on an ABI 3770 capillary sequencer (Applied Biosystems). To exclude cases of base misincorporation due to PCR error, haplotypes represented by only a single individual were verified by additional sequencing of an independent amplicon. The obtained sequences (480 bp) together with the homologous sequences of three outgroup species retrieved from the Genbank: *Pityogenes chalcographus* (DQ516014), *Ips cembrae* (AF113338) and *Ips pini* (AF113376) were aligned using Clustal X v1.83 (*18*) with the default settings. A maximum Parsimony (MP) approach was used as it is implemented in PAUP* version 4.0β (*19*), performing heuristic MP searches using 100 random-addition sequence replicates and exploring tree space by the Tree Bisection and Reconnection (TBR) branch swapping. MP bootstraps were performed using a heuristic search (100 random-addition-sequence replicates, TBR branch swapping) and 1000 pseudoreplicates. In addition to MP analysis, Bayesian analysis was performed by MrBayes version 3.1.1 (*20*) using the nucleotide substitution model proposed by MrModeltest v2.1 (*21*). General time-reversible model (GTR; *22*) with rate heterogeneity (*23*) using invariable sites (a=0.3969, I=0.4094) was found to be the most appropriate one. The number of generations was set to 10,000,000 with a sampling frequency of 100 generations in dual running process using four chains each run. After 2,207,000 generations, stationarity was achieved; the average standard deviation of split frequencies ranged between 0.001486 and 0.001328. Thus, only the last 7,793 trees of each run were used to compute a majority rule consensus tree and clade posterior probabilities.

RESULTS

Morphological data

The electron microscopic analysis of setal arrangement on antennal clubs or different setal types of the elytrae among *P. spinidens*, *P curvidens* and *P. vorontzowi* showed no differences in shape or length. Analysis of other parts of the beetle exoskeleton showed no new features useful for identification. The single obvious and consistent difference was the number of punctures in the

first and second interstria of the elytral disk. *P. spinidens* differed significantly from the other two species with fewer punctures, which varied to 5.9 ± 1.4(♂) and 6.0 ± 1.03(♀) for the first interstria and 6.2 ± 1.23(♂) and 6.8 ± 1.30 (♀) for the second interstria (Table 2). *P. curvidens* and *P. vorontzowi* had more similar punctures on the elytra compared to *P. spinidens* (Table 3, Figure 2). No difference was found in the number of punctures in the first and second stria comparing all three bark beetle species (Figure 2).

Molecular data

One specimen per species was analyzed and sequences were deposited in the Genbank (EF534717-EF534719). Four mutations occurred between *P. curvidens* and *P. vorontzowi*, three being on the 3rd codon position and one on the 1st codon position. The transition/transversion (Ts/Tv) ratio was 3/1. *P. curvidens* and *P. spinidens* revealed 79 differences, six on the 1st, three on the 2nd, and 70 on the 3rd codon position. Here the Ts/Tv ratio was 54/25 (Table 4). *P. vorontzowi* and *P. spinidens* revealed five 1st, three 2nd, and 68 3rd codon positions and a Ts/Tv ratio of 52/25 (Table 3).

Application of Bayesian statistics as well as of the MP criterion revealed that the three palearctic *Pityokteines* species are monophyletic (Figure 3). The posterior probability supporting the monophyly of the *Pityokteines* ge-

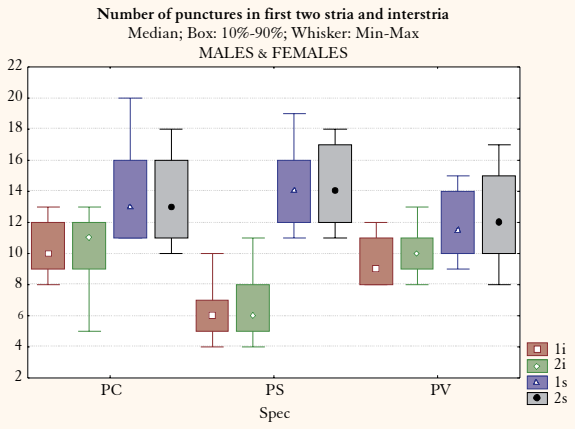

Figure 2. *Statistical analysis of the differences in the number of punctures in the 1st and 2nd striae and interstriae of the three sampled* Pityokteines *species.*

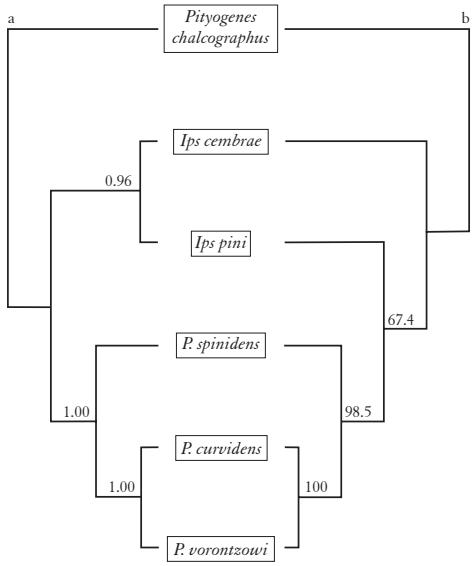

Figure 3. *a) 50% majority rule consensus tree constructed by Bayesian analysis and b) 50% majority rule consensus tree based on the Maximum Parsimony analysis, of the three* Pityokteines *species with three outgroup species. Numbers above nodes indicate posterior probabilities and bootstrap values (1000 replicates) for Bayesian and MP, respectively.*

TABLE 3

Multiple comparisons p values, Kruskal-Wallis test H(11, N=624)=419, p=0,000.

	Pc_m_1i	Pc_m_2i	Pc_f_1i	Pc_f_2i	Ps_m_1i	Ps_m_2i	Ps_f_1i	Ps_f_2i	Pv_m_1i	Pv_m_2i	Pv_f_1i	Pv_f_2i
Pc_m_1i					*	*	*	*				
Pc_m_2i					*	*	*	*	*			
Pc_f_1i					*	*	*	*				
Pc_f_2i					*	*	*	*	*			
Ps_m_1i	*	*	*	*					*	*	*	*
Ps_m_2i	*	*	*	*					*	*	*	*
Ps_f_1i	*	*	*	*					*	*	*	*
Ps_f_2i	*	*	*	*					*	*	*	*
Pv_m_1i		*		*	*	*	*	*				
Pv_m_2i					*	*	*	*				
Pv_f_1i					*	*	*	*				
Pv_f_2i					*	*	*	*				

Abbreviations: 1i – first interstriae, 2i – second interstriae, PC – *Pityokteines curvidens*, PS – *Pityokteines spinidens*, PV – *Pityokteines vorontzowi*

TABLE 4

In silico analysis of 480bp of the COI sequences. The total number and relative amount of mutational patterns observed in the CO1 gene were compared with expected values for mtDNA.

	P. curvidens vs *P. vorontzowi*		*P. spinidens* vs *P. vorontzowi*		P. *curvidens* vs *P. spinidens*		Expected value for mtDNA??[a]
	Absolute	Relative	Absolute	Relative	Absolute	Relative	
Total number of base substitutions	4	100	76	100	79	100	
1st codon position substitutions	1	25%	5	6.56%	6	7.6%	14.9 ± 9.4% [b]
2nd codon position substitutions	0	0%	3	3.95%	3	3.8%	4.5 ± 3.5% [b]
3rd codon position substitutions	3	75%	68	89.49%	70	88.6%	80.6 ± 21% [b]
Transitions/transversions	3/1		52/24		54/25		

[a] expected relative values as given in reference ± χ^2 confidence interval at $\alpha = 0.05$ (*25*)
[b] (*26*)

nus reached 1,00 and the bootstrap value was 98.5%. In both approaches, *P. curvidens* and *P. vorontzowi* were found to be sister species (1,00/100).

DISCUSSION

The existing taxonomic keys for the palearctic group of *Pityokteines* (*3, 10, 11*) are problematic as the average body size of two species is greater and ranges from 2.2 to 3.2 mm for *P. curvidens* and from 1.9 to 3.1 mm for *P. spinidens* while sizes for *P. vorontzowi* range between 1.9 to 2.5mm (*3, 12, 13*). Given that size range overlaps among the species, size is a poor identifier of species. Additional ambiguity appeared among the traditional qualitative characters used for species identification. For example, it has been reported that the shape outlined by the 2nd and 5th spines of the elytral declivity is square-like for *P. curvidens* and *P. vorontzovi* when compared to a rectangle shape for *P. vorontzovi* (*3*). In contrast, Urban (*13*) reports that the space between the spines for *P. curvidens* is rectangle-shaped while it is trapezoidal for the other species. In addition, the length of the setae on the anterior margin of the pronotum of females was an unreliable character for species determination. The hairs are often deformed, clumped and the length differences are unclear (*7*). Even the identification of males often lacks accuracy due to the fact that the position of the first sutural tubercle is not always such as described for the identification of *P. spinidens*.

This study found that the number of punctures in the first and second interstriae was a reliable character for identification of the three *Pityokteines* species (Table 3). Misidentification of *P. spinidens* by use of the mentioned characters was < 1% (Table 3, Figure 2). Since the character can be easily seen with a stereo microscope at 40x magnification, it is expected that this character can be utilized easily by forest entomologists.

The phylogenetic analysis provides an insight into the relationships of the three *Pityokteines* species. *P. curvidens and P. vorontzowi* are sister taxa, which was evident by both phylogenetic approaches (MP and Bayesian analysis) but was also revealed by nucleotide divergence – sequence divergence between *P. curvidens* and *P. vorontzowi* which was 0.83%. On the contrary, when these two species were compared with *P. spinidens*, sequence divergence was 16.46% compared to *P. curvidens* and 15.83% compared to *P. vorontzowi*) (Table 4). This result it makes evident that, besides being a novel morphological character, mtDNA markers represent a valuable tool that could facilitate the discrimination of *Pityokteines* species. However, the inclusion of more samples from these species will provide a more precise estimate of intra- as well as inter-specific differentiation and create the basis of a robust DNA barcoding tool for the palearctic *Pityokteines* species (*24*).

Acknowledgements: *Milan Pernek and Dimitrios N. Avtzis contributed equally to this study. We thank Željko Kauzlarić and Matija Pleše, Hrvatske šume d.o.o., Zagreb, Croatia, for their help in beetle sampling. Further we thank the Austrian Science Foundation (FWF) and the Austrian Exchange Service (OEAD) and the Austrian Ministry of Science for providing Milan Pernek the »Ernst Mach« fellowship used to conduct the presented research.*

REFERENCES

1. WOOD S 1982 The Bark and Ambrosia Beetles of North America (Coleoptera: Scolytidae): A Taxanomic Monograph. Great Basin Naturalist Memoirs, No 6, Brigham Young University, p 1359
2. KNIŽEK M 1998 A new species of Pityokteines (Coleoptera: Scolytidae) from Turkey. *Klapalekiana 34*: 189–193
3. PFEFFER A 1995 Zentral- und westpaläarktische Borken- und Kernkäfer. Naturhistorisches Museum, Basel, p 309
4. MAKSYMOV J 1950 Untersuchungen über den krummzähnigen Weißtannenborkenkäfer Ips curvidens Germ. während seiner Massenvermehrung 1947–49 in der Schweiz. *Mitteilungen der schweizerischen Anstalt für das forstliche Versuchswesen 26 (2)*: 499–584
5. POSTNER M 1974 Scolytidae (=Ipidae), Borkenkäfer. *In*: Schwenke W (*ed*) Die Forstschädlinge Europas, Bd. 2. Paul Parey, Hamburg, p 334–482
6. SCHWERDTFEGER F 1981 Waldkrankheiten. 4. Auflage. Paul Parey Verlag, Hamburg, Berlin, p 486
7. PERNEK M 2005 Natural enemies of the fir bark beetle genus Pityokteines (Col., Scolytidae) in Croatia with special emphasis on pathogens. PhD Thesis. Faculty of Forestry University of Zagreb, Zagreb, p 208

8. JURC M 2002 Najnevarnejši jelovi lubadarji (Pityokteines spinidens, P. curvidens, Cryphalus piceae) v Sloveniji. *Gozdarski Vestnik 60 (5–6)*: 259–265
9. KREHAN H, STEYRER G 2004 Borkenkäferkalamität 2003. *Forstschutz-Aktuell 31*: 6–12
10. REITTER E 1916 Fauna Germanica. Die Käfer des Deutschen Reiches. V. Band. K. G. Lutz Verlag, Stuttgart, p 343
11. GRÜNE S 1979 Brief Illustrated Key to European Bark Beetles. Verlag M. & H. Schaper, Hannover, p 182
12. ESCHERICH K 1923 Die Forstinsekten Mitteleuropas. 2. Band. Paul Parey, Berlin, p 663
13. URBAN J 2002 Diagnostic of bark beetles of the genus Pityokteines Fuchs important in forestry. *Journal of Forest Science 48*: 329–341
14. HEBERT P D N, STOECKLE M Y, ZEMLAK T S, FRANCIS C 2004 Identification of birds through DNA barcodes. *Plos Biology 10*: 1657–1663.
15. HEBERT P D N, PENTON E H, BURNS J M, JANZEN D H, HALLWACHS W 2004 Ten species in one: DBA barcoding reveals cryptic species in the neitropical skipper butterfly Astraptes fulgerator. *Proceedings of the National Academy of Science 10:* 14812–14817
16. LYNCH M, JARRELL P E 1993 A method for calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics 135*: 1197–1208
17. LUNT D H, ZHANG D X, SZYMURA J M, HEWITT G M 1996 The cytochrome oxidase I gene : evolutionary patterns and conserved primers for phylogenetic studies. *Insect Mol Biol 5*: 153–165
18. THOMPSON J D, GIBSON T J, PLEWNIAK F, JEANMOUGIN F, HIGGINS D G 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research 24:* 4876–4882
19. SWOFFORD D L 2001 PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4, Sinauer Associates. Sunderland, Massachusetts.
20. RONQUIST F, HUELSENBECK J P 2003 MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics 19*: 1572–1574
21. NYLANDER J A A 2004 MRMODELTEST 2.1. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
22. RODRIGUES F, OLIVER J F, MARIN A, MEDINA J R 1990 The general stochastic model of nucleotide substitutions. *J Theor Biol 142*: 485–501
23. YANG Z 1993 Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Mol Biol Evol 10*: 1396–1401
24. COGNATO A I, SUN J H 2007 DNA based cladograms augment the discovery of a new Ips species from China (Coleoptera: Curculionidae: Scolytinae). *Cladistics 23*: 539–551
25. SACHS L 1999 Angewandte Statistik. Springer Verlag, Berlin, p 884
26. BLOUIN M S, YOWELL C A, COURTNEY C H, DAME J B 1998 Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Mol Biol Evol 15*: 1719–1727