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Genetic and Morphological Comparisons of New and Old World Populations of *Spalangia* Species (Hymenoptera: Pteromalidae)

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ABSTRACT The genetic similarity of New and Old World samples of *Spalangia* spp. (Hymenoptera: Pteromalidae) was examined using two ribosomal DNA regions. The species examined were *Spalangia cameroni* Perkins, *Spalangia endius* Walker, *Spalangia gemina* Bouček, *Spalangia nigra* Latreille, *Spalangia nigroaenea* Curtis, and *Spalangia slovaca* Bouček. Two species of *Muscidifurax*, *Muscidifurax raptor* Girault & Sanders and *Muscidifurax zaraptor* Kogan & Legner (Hymenoptera: Pteromalidae) were included as outgroup taxa. The internal transcribed spacer (ITS)-1 region was highly variable among *Spalangia* species with many insertions/deletions making alignment of the sequences difficult. The D2-D3 region of the 28s ribosomal gene and the nuclear rDNA 18s gene were more conserved and enabled phylogenetic analysis. No genetic differentiation was observed among *S. cameroni* and *S. endius* samples from Kazakhstan, Russia, and North America. New World samples of *S. nigroaenea* are genetically distinct from *S. slovaca*, a morphologically similar Old World species that is newly recorded from Kazakhstan and Russia. The intact 920 bp ITS-1 amplicon of *S. nigroaenea* was much larger than the 780-bp amplicon of *S. slovaca*. Kimura two-parameter genetic distance between the two species was 0.015 for the 28s region. Otherwise, the smallest genetic distance among recognized *Spalangia* species was 0.037 between *S. endius* and *S. nigra*. The genetic distance between *M. raptor* and *M. zaraptor* was 0.004. Based upon these results, the utility of the D2-D3 region of the 28s ribosomal gene is substantiated for differentiating species of *Spalangia*. The molecular analysis of the six *Spalangia* species revealed two groupings: *S. nigroaenea* and *S. slovaca* and *S. cameroni* and *S. gemina*. A third clade, *S. endius* and *S. nigra*, was observed, but bootstrap support was weak. These relationships were compared with those indicated by morphology and all agreed except possibly between *S. endius* and *S. nigra*, for which morphological evidence is equivocal. Morphological features are described and illustrated to distinguish the morphologically most similar species, *S. nigra*, *S. nigroaenea*, and *S. slovaca*, from each other and from other *Spalangia* spp.

KEY WORDS molecular diagnostics, *Spalangia*, filth fly parasitoids, Pteromalidae

Wasps in the genus *Spalangia* Latreille (Hymenoptera: Pteromalidae) are frequently observed parasitoids of house flies and stable flies (Legner et al. 1967, Petersen and Meyer 1983, Jones and Weinzierl 1997), and many are pupal parasitoids of filth flies in the confined livestock environment (Miller and Rutz 1990, Petersen et al. 1990). Most of the *Spalangia* species found in North America have cosmopolitan distributions, being found also in Europe, Asia, and the Pacific regions. However, much of the present day distribution of these wasps is probably secondary, having either been intentionally introduced to control pest flies or simply accompanied such flies in their range expansions around the world. Given the broad distribution of these wasps, several species have been described from disparate geographic regions and subsequently synonymized (Bouček 1963). Phylogenetic relationships among *Spalangia* species have yet to be investigated rigorously, and relationships are poorly known, although Bouček (1963) divided the 14 Holarctic species recognized into four species groups based on morphological similarity. In this study, we examine the genetic similarity of North American, European, and Asian samples of six *Spalangia* species—*Spalangia cameroni* Perkins, *Spalangia endius* Walker, *Spalangia gemina* Bouček, *Spalangia nigra* Latreille, *Spalangia nigroaenea* Curtis, and *Spalangia slovaca* Bouček—and compare indicated relationships to those suggested by morphology within the context of the species-groups established by Bouček (1963).

Eucaryote nuclear rDNA is organized as tandem repeats with copy numbers up to 5000 per genome. Each repeat consists of three genes, 18s, 5.8s and 28s, and three spacer regions, the intergenic spacer (IGS), and two internal transcribed spacers (ITS), ITS-1 be-

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tween the 18s and 5.8s and ITS-2 between the 5.8s and 28s genes. The high copy number makes rDNA easier to amplify than single copy genes, but variation among copies can be problematic (Hwang and Kim 1999). In this study, we examined two nuclear ribosomal sequences. ITS-1 has been used to differentiate species and closely related populations (Pfeifer et al. 1995, Powers et al. 1997, Szalanski et al. 1997, Taylor and Szalanski 1999). This region diverges at a rate 3–4 times faster than mitochondrial DNA in reproductively isolated populations, but, in general, the repeats remain relatively homogeneous within individuals and populations (Zimmer et al. 1980, Elder and Turner 1995). Rapid divergence and concerted evolution make the ITS region ideal for molecular diagnostics. The 28s rDNA gene has proven useful for species diagnostics and molecular systematics of parasitic Hymenoptera (Campbell et al. 2000) among other organisms. This gene is more conserved that the ITS-1 region (Hwang and Kim 1999).

The objective of this study was to determine the phylogenetic relationships between New and Old World populations of Holarctic *Spalangia* species as well as the relationships among *Spalangia* species. Morphological features are newly described and illustrated to assist recognition of *S. slovaca.* A secondary goal was to develop markers for using molecular techniques to differentiate the more common *Spalangia* species.

Materials and Methods

Techniques used for this study were similar to those used by Taylor and Szalanski (1999). DNA was isolated from frozen and alcohol-preserved specimens by using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). The ITS-1 region was amplified with the forward primer $rDNA_2$ (TTGATTACGTCCCTGCCCTTT; Vrain et al. 1992) and one of three reverse primers: 1r, 35r (GTGATCCACCGTTCAGGGTA and AGCTGGCTGCGTTCTTCATCGA, respectively; Ratcliffe et al. 2002) or $rDNA_{1.58s}$ (ACGAGCCGAGTGATCCACCG; Cherry et al. 1997). The D2 and D3 regions of the 28s ribosomal gene were amplified with the primers D2f and D2r (GCGAACAAGTACCGTGAGGGG and TAGTTCACCATCTTTCGGGTC, respectively; Belshaw and Quicke 2002). The polymerase chain reaction (PCR) protocol was 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplification products were cleaned with Micron YM50 filters (Millipore Corporation, Billerica, MA) and sequenced by the Kansas State University Sequencing Facility (Manhattan KS). Sequences were aligned with BioEdit 5.09 (Hall 1999) and CLUSTAL W (Thompson et al. 1994). ITS-1 sequences were truncated to the 3′ end of the 1r primer for comparisons.

Muscidifurax raptor Girault & Sanders and *Muscidifurax zaraptor* Kogan & Legner (Hymenoptera: Pteromalidae) were used as the outgroup taxa for the phylogenetic analysis of the 28s data set, and *M. raptor* was the outgroup taxon for the 18s data set. Maximum likelihood and unweighted parsimony analysis on the alignments were conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing data. The reliability of trees was tested with a bootstrap test (Felsenstein 1985). Parsimony bootstrap analysis included 1,000 resamplings by using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the default likelihood parameter settings were used (HKY85 six-parameter model of nucleotide substitution, empirical base frequencies, and transition/transversion ratio set to 0.917:1 for the 18s data set, and 1.540 for the 28s data set). These parameters were used to carry out a heuristic search using PAUP*, by using either the single most parsimonious tree as the starting tree, or stepwise addition.

The samples used for this study are listed in Table 1. Vouchers of the species we identify as *S. slovaca* are deposited in the Canadian National Collection of Insects (Ottawa, Ontario, Canada) and vouchers of all the species are deposited in the Nebraska State Museum Entomology (Lincoln, NE) collection. All sequences have been submitted to GenBank (AY855172–AY855208 for the 28s rDNA amplicon and DQ411978–DQ412041 for the ITS-1 amplicon). Morphological terminology follows Bouček (1963) and Gibson (1997).

Table 1. Strains of *Spalangia* spp.

Species	*n*	Origin	Yr collected
S. cameroni	2	Arkansas[a]	2004
	2	Florida[b]	2003
	2	Minnesota[c]	1999
	2	Nebraska[d]	2001
	2	New York[e]	2002
	3	Kazakhstan[f]	1999
	2	Russia[g]	1999
S. endius	2	Arkansas[a]	2004
	2	Florida[b]	2003
	2	Minnesota[c]	1999
	2	Nebraska[d]	2002
	2	Kazakhstan[f]	1999
	2	Russia[g]	1999
S. gemina	2	Brazil[h]	1991
S. nigra	2	Minnesota[c]	2002
	3	Nebraska[d]	2002
S. nigroaenea	2	Florida[b]	2003
	2	Minnesota[c]	2002
	4	Nebraska[d]	2001, 2002
S. slovaca	2	Kazakhstan[f]	1999
	2	Russia[g]	1999

[a] University of Arkansas, Fayetteville, AR, 36° 18′ N, 95° 25′ W.
[b] USDA–ARS, Gainesville, FL, 29° 47′ N, 82° 29′ W.
[c] University of Minnesota, Minneapolis, MN, 44° 57′ N, 98° 28′ W.
[d] Midwest Livestock Insect Research Laboratory, USDA–ARS, Lincoln, NE, 41° 10′ N, 96° 28′ W
[e] Cornell University, Ithaca, NY, 42° 26′ N, 76° 25′ W.
[f] Colony maintained at USDA–ARS, Gainesville FL, pooled from nine locations in the vicinity of Almaty, 43° 10′ N, 77° E; 43° 10′ N, 76° 50′ E; 43° 20′ N, 77° 10′ E; 43° 40′ N, 78° E; 43° 30′ N, 76° 30′E; 43° 10′ N, 76° 50′ E; 43° 20′ N, 77° 10′ E; 43° 20′ N, 77° 15′ E; and 43° 40′ N, 77° 30′ E.
[g] Colony maintained at USDA–ARS, Gainesville FL, pooled from seven locations in the vicinity of Kropotkin and Maikop, 45° 20′ N, 41° E; 45° 20′ N, 41° E; 45° 20′ N, 40° 30′ E; 45° 15′ N, 40° 25′ E; 45° 20′ N, 40° 30′ E; 44° 10′ N, 40° E; and 45° 5′ N, 40° 10′E.
[h] Colony maintained at USDA–ARS, Gainesville FL, collected near Sao Paulo, Brazil.

```
slovaca      1   CGCTACTACC GATTGAATGA TTTAGTGAGG TCTTCGGACC GGTGCGCGGC AATGTCTTCT
nigroaenea   1   ....C..... .......... .......... .......... .......... .........A

slovaca      61  TAGCATTGCC GATTGTGCTG GGAAGATGAC CAAACTTGAT CATTTAGAGG AAGTAAAAGT
nigroaenea   61  .......... .......... .......... .......... .......... ..........

slovaca      121 CGTAACAAGG TTTCCGTAGG TGAACCTGCG GAAGGATCAT TAACGTATGC G--CAACTAA
nigroaenea   121 .......... .......... .......... .......... .......... .AA..TA.G.

slovaca      179 AACGCAACGT CTTTTAAATC GTTCCTCGCG AACGATCAAA A--------- --CTATA---
nigroaenea   181 .......... ...GC.--.. .......... .......... .AATAAAAGG TGTG...ATC

Slovaca      224 --TTCTGAAC TTGT-GCCCC CGTCCGTGCG TATCTTGCGC GCGGATTGGG GGGGCTCGTT
nigroaenea   239 CTC....... ..C.T.T.T. G..T..C.T. .-C.C..G.G ...CGCGA.T ..A.......

slovaca      282 CGGG------ ---------- ------CCTC GAATGTGCAT TGAAAATGCC GGATCGATCG
nigroaenea   298 ...AACACAC AGAGGAGAAA CGCACCT... ....A..... C.....C... ..........

slovaca      320 GACGGTACGC GGCTGCCTTG TATCATCGTA CATGGCTGTC CGCTTCATCC ATTGATTCGG
nigroaenea   358 .......... ........CT C.-------- -.......A. .......... ..CTT.....

slovaca      380 TGCGTAGTCT CGCTGTGGCA ACCTACTCCT CT-------- ---------T CTCAGTGTCA
nigroaenea   409 ....C..... .......... .......... ..ACTCCTCT CTCTCTCTC. T.........

Slovaca      423 ACGCAGTCAC GC-------- ---------- -----GAGAG AGGGGCGATC GTC-TTGTAC
nigroaenea   469 .......... .ATGGGAGAA AAAGGGAGTG TGTGA..C.. .......... ...G.C....

slovaca      459 GA-------T CGATGGTTTT TGAATGAATC CACGCCCGTT CAGTCGAGAT TGCGCCATCG
nigroaenea   529 ..ATGATGA. .......... .......... .......... .......... ....T.....

slovaca      512 TGCTCGCTTT ACACGAAGCG TGCGATGCGA CGCCTTTAGT A----ACTAT TTT-------
nigroaenea   589 .--..... T......... A...G..... .......... CCGTT..CG. CG.CTTGATC

slovaca      560 ---------- -GGTAACTTT GGGCCGACGA CGAGGGCAAT A--------- ------CGCT
nigroaenea   647 GACGGTCGCG C...T..... .......... .T..A..CGC GTGTGTTATA TACGCG....

slovaca      595 CGAATCGTTG AATACGTACC GTCTGGTCGG CGCCGCTGTT TAGACTTGTG CTATCGGCGA
nigroaenea   707 .......... ......A..T .......... .......... .C.C.C.... .G.....T.T

slovaca      655 GCGCTTGACT ATTTTTGAAT GCTTTGTTTT AAAATGCAAT GCGTAAAAAA TATTAAATGA
nigroaenea   767 .......... -.A....... .......G.. .......... .......... ......-...

slovaca      715 T
nigroaenea   825 .
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Fig. 1. ITS-1 sequences for *S. nigroaenea* and *S. slovaca.* A "." indicates identity, "-" indicates an insertion/deletion.

Results

Molecular Analysis. The ITS-1 amplicon varied greatly among the six *Spalangia* species in this study. The length of the intact amplicon varied from 655 bp in *S. nigra* to 922 bp in *S. nigroaenea.* High levels of interspecific differentiation made alignment of the sequences for phylogenetic analysis difficult. Samples of *S. cameroni* and *S. endius* from Russia, Kazakhstan, and the United States revealed little intraspecific variation. Two single-base polymorphisms, a T/G at base 310 and C/A at base 371, were observed in *S. cameroni* from Russia and Kazakhstan. Four of five wasps from Russia were G/A (310/371) for these polymorphisms, and the fifth was G/C. Two of four wasps from Kazakhstan were G/A, and the other two were T/C. All North American *S. cameroni* were T/C. The polymorphism in base 371 was in a TaqI restriction site and was verified in restriction digests. No variation was observed among *S. endius* from Russia, Kazakhstan, Arkansas, Florida, Minnesota, or Nebraska. The ITS-1 amplicon of *S. nigroaenea* differed significantly from that of *S. slovaca.* The *S. nigroaenea* amplicon was 920 bp compared with 780 bp for *S. slovaca* (Fig. 1). Seven insertion/deletions of 7–23 bp each plus smaller insertion/deletions of 1–4 bp and substitutions were diagnostic for the two species. No variation was observed within either of the two species.

Although the ITS-1 region was too variable to align for phylogenetic analyses, this amplicon contained 164 bp of the 3′ end of the 18s rDNA gene. Kimura two-parameter genetic distances among and within species are presented in Table 2. The 3′ end of the 18s gene did not differ between *S. nigroaenea* and *S. slovaca.* Other interspecific genetic distances varied from 0.006 between *S. endius* and *S. nigra* to 0.046 between *S. nigroaenea* and *S. nigra.* Intergeneric genetic distances were between 0.053 and 0.067. The average base frequencies for the 18s gene were A = 0.29, C = 0.18, G = 0.26, and T = 0.28. The aligned 18s DNA data matrix, including the outgroup taxa resulted in a total of 161 characters. Of these characters, 18 (11%) were variable, and 12 (7%) were phylogenetically informative. This data set had only one most parsimonious tree (length = 23, CI = 0.957), as documented using the

Table 2. Genetic distance among *Spalangia* spp. 18s ribosomal gene (Kimura two-parameter model)

	S. cameroni	*S. endius*	*S. slovaca*	*S. nigroaenea*	*S. gemina*	*S. nigra*	*M. raptor*
S. cameroni	<0.006	0.033	0.026	0.026	0.013	0.033	0.067
S. Endius		0.000	0.040	0.039	0.026	0.006	0.053
S. slovaca			0.000	0.000	0.013	0.046	0.054
S. nigroaenea				0.000	0.013	0.046	0.054
S. gemina					0.000	0.026	0.053
S. nigra						0.000	0.060

Branch and Bound search algorithm of PAUP*. Bootstrap analysis of the aligned *Spalangia* and the outgroup taxon resulted in a consensus tree with three distinct branches. These clades included *S. cameroni* and *S. gemina*, *S. endius* and *S. nigra*, and *S. nigroaenea* and *S. slovaca*. Regardless of whether the starting tree was the most parsimonious tree or was obtained via stepwise addition, the maximum likelihood search found only one tree ($-\ln L = 1953.966$). The maximum likelihood tree was identical to the maximum parsimony tree.

The D2-D3 amplicon of the 28s rDNA gene was ≈765 bp, of which 680 bp were reliably sequenced in all of the wasp species and used for analysis. The 28s region was much more conserved than ITS-1. Divergence among recognized *Spalangia* species was 0.037–0.100, whereas that between *M. raptor* and *M. zaraptor* was 0.004. Divergence between *Spalangia* and *Muscidifurax* was ≤0.159 (Table 3). The D2-D3 region was slightly G-C biased in both *Spalangia* and *Muscidifurax*, 57 and 60%, respectively. No differentiation between Old and New World populations of either *S. cameroni* or *S. endius* was observed. Two polymorphic nucleotides were observed in *S. cameroni*. One wasp from Nebraska had a C at base 163, whereas all of the others had a T at that position. Review of the chromatogram indicated that this wasp was polymorphic with both C and T versions present. One *S. cameroni* from Russia had an A at base 653, whereas all of the rest of the *S. cameroni*, including those from Kazakhstan, had a T at this site. Examination of the chromatograms indicated that both *S. cameroni* from Russia were polymorphic at this site as were the two *S. cameroni* from Nebraska. *S. cameroni* from New York and Kazakhstan seemed to be fixed for T at this position. No variation was observed among New and Old World *S. endius*. *S. nigroaenea* and *S. slovaca* were clearly divergent. Twelve of 680 nucleotide sites were diagnostic for the two species, whereas no variation was observed within either species.

The aligned 28s DNA data matrix, including the outgroup taxa resulted in a total of 678 characters. Of these characters, 151 (22%) were variable and 147 (22%) were phylogenetically informative. This data set had only one most parsimonious tree (length = 204, CI = 0.843), as documented using the Branch and Bound search algorithm of PAUP*. Bootstrap analysis of the aligned *Spalangia* and the outgroup taxa indicates that *S. cameroni* and *S. gemina* are sister species as are *S. nigroaenea* and *S. slovaca* (Fig. 2). A third clade, *S. endius* and *S. nigra*, had poor support in the bootstrap analysis, 51%. Distinct clades were not observed among New and Old World populations of either *S. cameroni* or *S. endius*. Regardless of whether the starting tree was the most parsimonious tree or was obtained via stepwise addition, the maximum likelihood search found only one tree ($-\ln L = 2658.754$). The maximum likelihood tree was identical to the maximum parsimony tree.

Although morphological differentiation of *Spalangia* species is easier than for *Muscidifurax* species, it can still be difficult, especially for those not familiar with the characters or for those trying to identify immature parasitoids within the host puparium. Restriction enzyme digests of the ITS-1 amplicon were used to easily differentiate all of the *Spalangia* species in this study, including *S. slovaca* and *S. nigroaenea* (Table 4). Although the fragment sizes presented are from simulated digests based upon the sequences (Webcutter 2.0; Heiman 1997), all digests were verified.

Morphological Analysis. Virtually no information is available concerning interspecific relationships among *Spalangia* species. Bouček (1963) treated 45 world species. Of these species, Bouček segregated into four species-groups the 14 species recognized from the Holarctic region. The "*nigra*" group consisted of *S. nigra*, *Spalangia irregularis* Bouček, and *Spalangia rugulosa* Förster and included species with dense coarse sculpture, the pronotum without any distinct,

Table 3. Genetic distance among *Spalangia* spp. D2-D3 regions of 28s ribosomal gene (Kimura two-parameter model)

	S. cameroni	*S. endius*	*S. slovaca*	*S. nigroaenea*	*S. gemina*	*S. nigra*	*M. raptor*	*M. zaraptor*
S. cameroni	<0.003	0.092	0.100	0.100	0.067	0.066	0.154	0.152
S. endius		0.000	0.080	0.083	0.082	0.037	0.141	0.137
S. slovaca			0.000	0.015	0.095	0.064	0.159	0.157
S. nigroaenea				0.000	0.096	0.063	0.162	0.161
S. gemina					0.000	0.062	0.149	0.148
S. nigra						<0.002	0.148	0.148
M. raptor								0.004

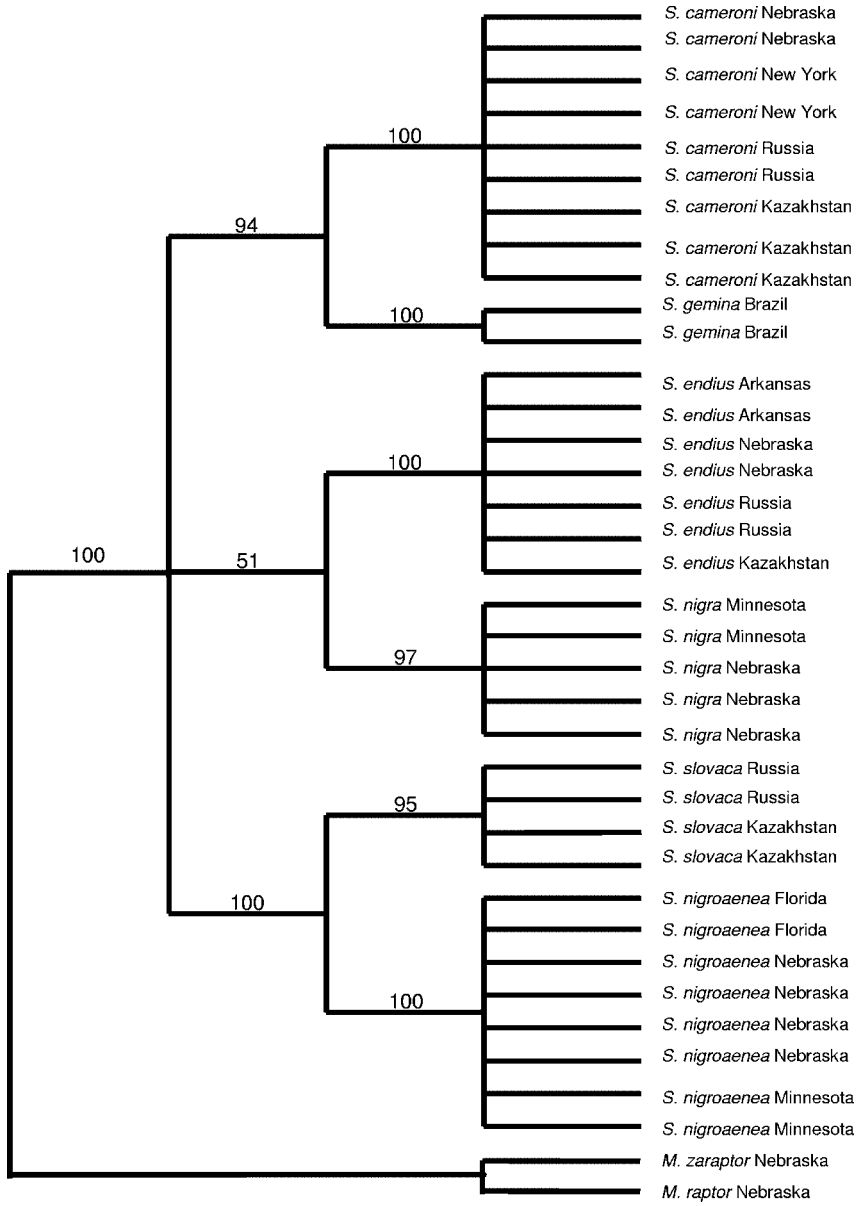


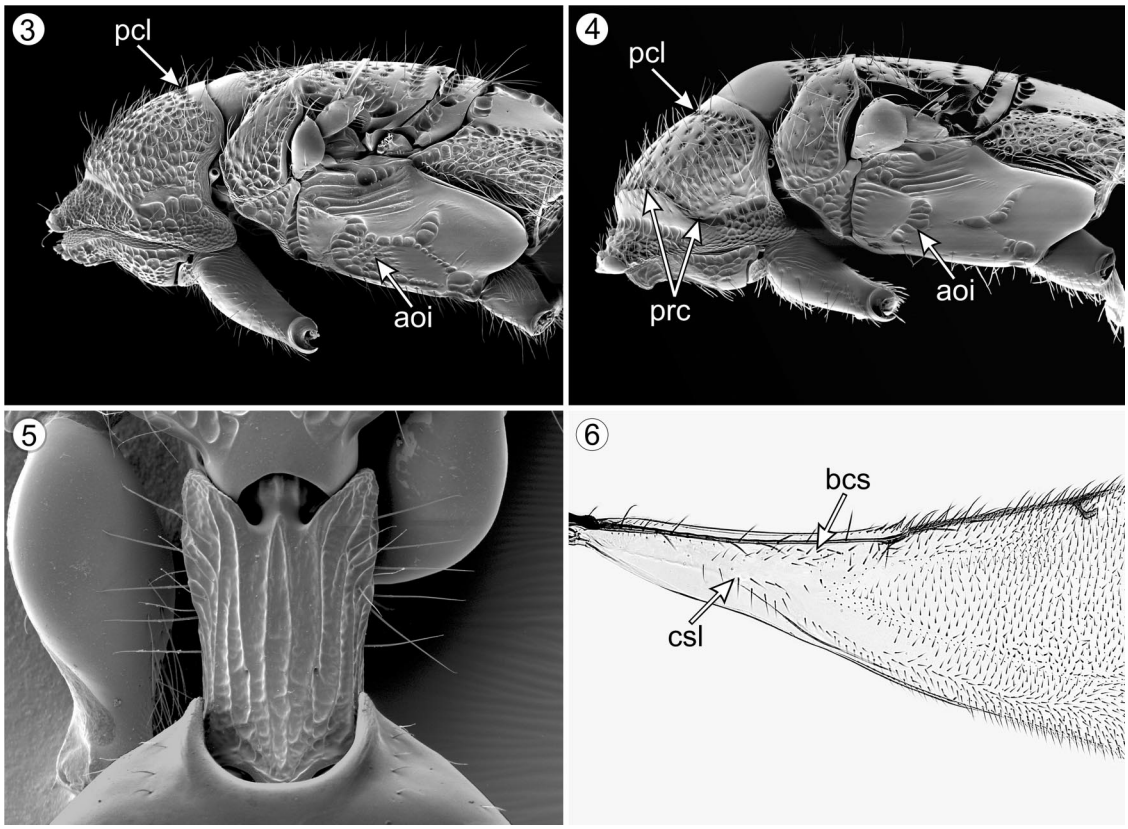
Fig. 2. Maximum parsimony tree of *Spalangia* spp. based upon 680 bp of the 28s D2-D3 region of rDNA.

isolated cross-line of punctures. The "*nigroaenea*" group consisted of *S. cameroni*, *S. endius*, *S. gemina*, *S. nigroaenea*, and *S. slovaca* and included species that had a distinct pronotal cross-line. An unnamed group, consisting of *Spalangia crassicornis* Bouček, *Spalangia erythromera* Förster, and *S. nigripes*, included those

Table 4. Restriction fragment lengths of ITS-1 amplicon based upon simulated digests (Webcutter) for *Spalangia* spp

Restriction enzyme	*S. cameroni*	*S. endius*	*S. gemina*	*S. nigroaenea*	*S. nigra*	*S. slovaca*
Uncut	734	730	741	922	655	785
MseI	299, 235, 194, 6	259, 197, 180, 94	287, 194, 173, 87	634, 197, 67, 24	232, 226, 197	491, 196, 42, 32, 24
TaqI	232, 230, (101, 49), 64, 58	263, 141, 124, 70, 60, 38, 34	304, 297, 116, 24	363, 187, 179, 73, 61, 34, 14, 11	262, 221, 153, 19	324, 155, 148, 99, 34, 25

Variable sites are indicated parenthetically.



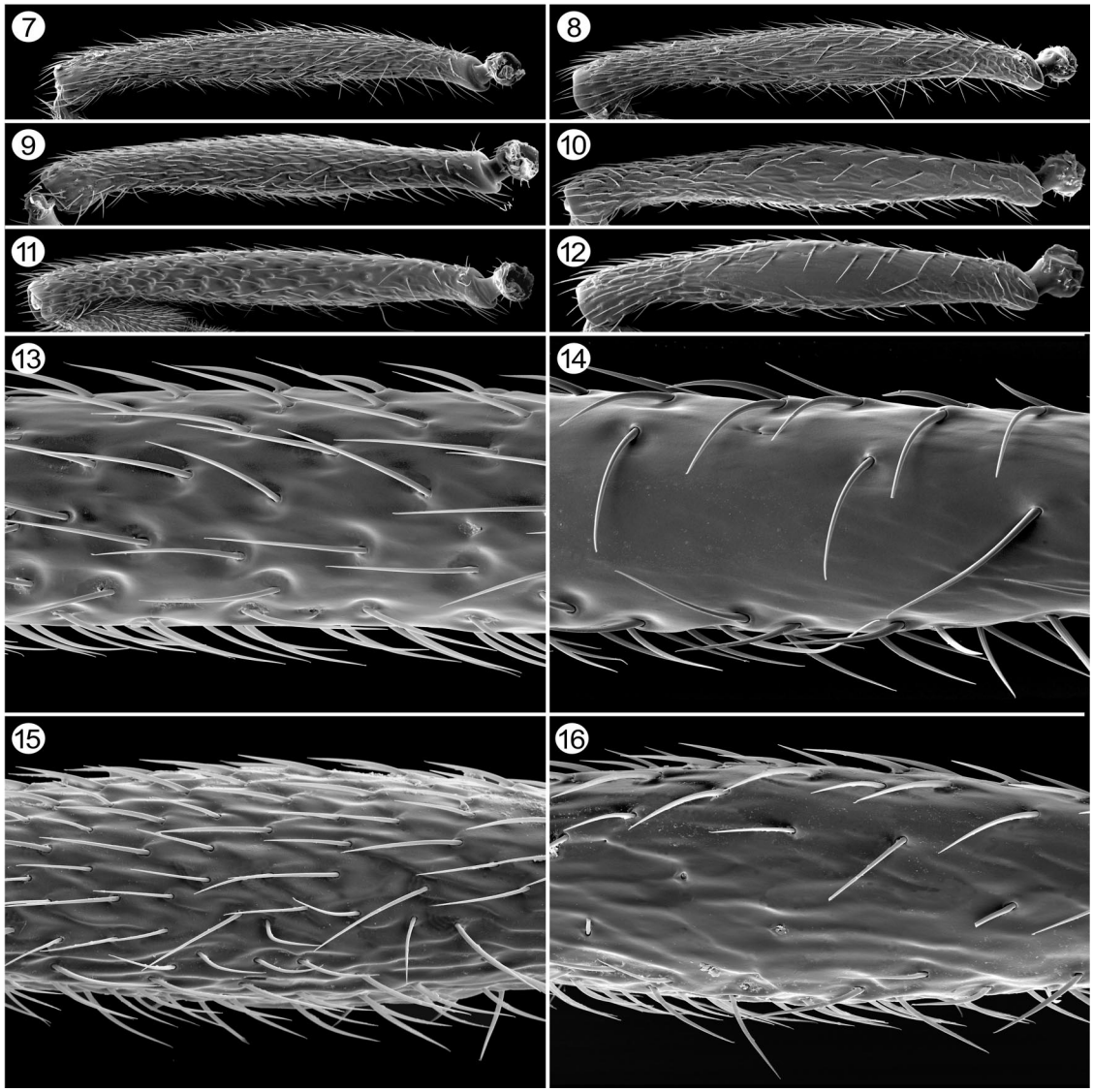
Figs. 3–6. Figs. 3 and 4, lateral mesosoma: 3, *S. nigra*; 4, *S. nigroaenea*. Figs. 5 and 6, *S. nigra*: 5, petiole; 6, forewing (aoi, anterior oblique impression; bcs, basal cell setae; csl, costal setal line; pcl, pronotal cross-line; and prc, pronotal carina).

species without a distinct pronotal cross-line but with a developed frenal line on the scutellum. Finally, the "*fuscipes*" group, consisting of *S. drosophilae*, *Spalangia fuscipes* Nees, *S. hematobiae*, and *Spalangia subpunctata* Förster, included species with the frenal line weak or lacking. Although these species-groups reflect a general reduction in sculpture from the *nigra*-group to the *fuscipes*-group, they are based on "key" characters that do not necessarily reflect phylogenetic relationships. For example, of the three species assigned to the *nigra*-group, *S. irregularis* and *S. rugulosa* have an almost completely rugose pronotum, because the depressions are formed by irregular linear ridges (Bouček 1963, figs. 8 and 12), whereas in *S. nigra* the pronotum is mostly punctate because the individual depressions, although crowded, are more distinctly separate and circular (Bouček 1963, fig. 14). This "punctuate" type of sculpture is more similar to *S. nigroaenea*, *S. slovaca*, and *S. endius* within the *nigroaenea*-group (Bouček 1963, figs. 17, 23, 30) as well as many other *Spalangia* species. Although *S. nigra* was included in the *nigra*-group because it lacks a distinct pronotal cross-line, it does indeed have a uniformly developed cross-line of punctures near the posterior margin of the pronotum. It is only because the pronotum is more extensively and closely punctate in *S. nigra* that the cross-line (Fig. 3, pcl) is less distinct than for *S. nigroaenea* (Fig. 4, pcl) and other *nigroaenea*-group species. Furthermore, the rugose pronotal sculpture of *S. irregularis* and *S. rugulosa* is similar to the pronotal sculpture of *S. cameroni* and *S. gemina* within the *nigroaenea*-group, except that in the latter two species the sculpture is effaced medially so that the posterior line of punctures is distinct (Bouček 1963, fig. 26). Consequently, depending on polarity of pronotal sculpture, i.e., punctate → rugose or rugose → punctate, and possible character transformation within these, e.g., entirely rugose → partly rugose, *S. nigra* could be more closely related to *S. nigroaenea*, *S. slovaca* and *S. endius*, whereas *S. irregularis* and *S. rugulosa* could be more closely related to *S. cameroni* and *S. gemina*.

Of the five species assigned to the *nigroaenea*-group, both sexes of *S. nigroaenea* and *S. slovaca* have a conspicuously setose petiole, with several long white setae projecting from either side over at least its anterior half (Fig. 5; Bouček 1963, fig. 76), whereas *S. endius*, *S. cameroni*, and *S. gemina* have the petiole bare or with only one or two short setae projecting dorsally or from either side. A conspicuously setose petiole is also shared with both sexes of all *nigra*-group species except possibly for *S. irregularis*, of which we have seen only a single female and male paratype. The female has a conspicuously setose petiole like other members of

the group, but the male has only three long setae projecting from one side near its middle. This asymmetry probably results from abrasion of the setae from one side, but additional specimens are required to determine extent and variability of the petiolar setae in *S. irregularis* males. Both sexes of the Neotropical species *Spalangia chontalensis* Cameron also have a conspicuously setose petiole (Bouček 1963, fig. 76) and a punctate pronotum (Bouček 1963, fig. 77) that would place the species within the *nigroaenea*-group sensu Bouček. Other species that we know from at least the Nearctic, Palearctic and Neotropical regions have the petiole bare or with only one or two short setae projecting dorsally or from each side, except for *S. fuscipes.* At least some females and males of this species have three or four long setae projecting laterally and dorsally from near the middle of the petiole, similar to the male of *S. irregularis.* However, *S. fuscipes* is undoubtedly more distantly related to the former group of species based on a different habitus and sculptural features, including much finer pronotal and scutellar sculpture (Bouček 1963, fig. 52) and a different form of mesopleural sculpture. This character-state distribution indicates that a setose petiole is either the groundplan state for *Spalangia* and the setae have been lost independently in different lineages, or that they have been derived independently in at least two lineages. This cannot be resolved without a comprehensive phylogenetic analysis. If a conspicuously setose petiole is apomorphic, then *S. irregularis*, *S. rugulosa*, *S. nigra*, *S. nigroaenea*, *S. slovaca*, and *S. chontalensis* are indicated to comprise a monophyletic group within *Spalangia*, which would not support the *S. nigra* + *S. endius* relationship indicated by genetic similarity (Tables 2 and 3; Fig. 2).

S. nigra is one of four species, including *S. nigroaenea*, *S. slovaca*, and *S. chontalensis* that share both a coarsely punctate (rather than rugose) pronotum and a conspicuously setose petiole, but it differs from the other three species in several features. Like most other species of *Spalangia*, *S. nigra* has the pronotum rounded anteriorly, the sculpture being uniformly punctate-alveolate posterior to the crenulate furrow that differentiates the neck from the collar (Fig. 3). Individuals of the other three species have a variably distinct pronotal carina (Fig. 4, prc) dorsally that separates an anterior, more vertical portion of the collar from the larger horizontal portion of the collar. Even if the anterior pronotal carina is indistinct dorsally, anterolaterally there is a distinct vertical carina on the side of the pronotum. This uniquely shared feature indicates that *S. chontalensis, S. nigroaenea* and *S. slovaca* comprise a monophyletic group, and supports the relationship between *S. nigroaenea* and *S. slovaca* indicated by genetic distance. *S. nigra* also has a large, subtriangular "anterior oblique impression" sensu Bouček (1963) (fig. 3), the sculptured region expanding ventrally and extending broadly along the transepisternal ridge (Fig. 3, aoi), which is similar to the mesopleural sculpture patterns of the two other *nigra*-group species, but also *S. endius* within the *nigroaenea*-group. *S. chontalensis*, *S. nigroaenea*, and *S. slovaca* have a more or less vertical, lunate-to-semicircular anterior oblique impression (Fig. 4, aoi; Bouček 1963, fig. 3), which is more similar to the sculpture patterns of *S. cameroni* and *S. gemina.* Both sexes of all three *nigra*-group species and *S. chontalensis* also have a line or lines of setae on the dorsal surface of the forewing along the cubital and basal folds as well as some setae within the basal cell (Fig. 6, bcs, csl), whereas females of *S. nigroaenea* lack these setae or have only 1–3 scattered setae near the apex of the basal cell, and both sexes of *S. slovaca* lack the setae. Presence or absence of the setae is also variable in other *Spalangia.* For example, males of *S. endius* have the setae, whereas females usually lack the setae, and both sexes of *S. cameroni* and *S. gemina* lack the setae, which indicates independent loss or gain of the setae in different *Spalangia* lineages. However, if polarity is from presence → absence of setae for *S. chontalensis* + *S. nigroaenea* + *S. slovaca*, then the former species is indicated as the sister species of the latter two species, the setae being lost from females of the immediate ancestor of *S. nigroaenea* + *S. slovaca* and subsequently also from the males of *S. slovaca.* Regardless, the presence of setae readily differentiates both sexes of *S. chontalensis* from those of *S. slovaca*, and males of *S. nigroaenea* from males of *S. slovaca*, but not females of *S. nigroaenea* and *S. slovaca.* Bouček (1963) differentiated females of *S. nigroaenea* and *S. slovaca* based on comparatively subtle features. Compared with *S. slovaca*, *S. nigroaenea* was stated to have a subpentagonal versus subglobose pronotal collar (Bouček 1963, cf. figs 17, 23), oblong versus subquadrate distal funicular segments (Bouček 1963, cf. figs 18, 22), gena longer versus shorter than eye length, and head oblong versus hardly longer than broad (Bouček 1963, cf. figs 19, 20 with 21), although Bouček noted that head structure was variable in *S. nigroaenea* (Bouček 1963, cf. figs 19, 20). Females of *S. nigroaenea* differ more conspicuously from *S. slovaca* in sculpture of the antennal scape. The outer surface of the scape of female *S. nigroaenea* is uniformly covered with distinct, separate, setiferous punctures (Figs. 11 and 13), whereas the inner surface is more or less longitudinally strigose apically, but it has a conspicuous, bare, smooth and shiny longitudinal region over at least its basal half (Figs. 12 and 14). Females of *S. slovaca* lack distinct setiferous punctures on the outer surface of the scape, the setae arising from very shallow and more closely crowded punctures, so that the surface seems rougher and less shiny than in *S. nigroaenea*, with the ridge-like interstices forming "somewhat longitudinally" arranged sculpture (Figs. 9 and 15) as was described by Bouček (1963). The inner surface of the scape is similar to that of *S. nigroaenea* except that the bare region is at least finely (Figs. 10 and 16) and sometimes distinctly longitudinally striate. Females of *S. chontalensis* have both the outer (Fig. 7) and inner (Fig. 8) surfaces distinctly, longitudinally striate, similar to such species as *S. nigra* and *S. endius.* If *S. chontalensis* is the sister species of *S. nigroaenea* + *S. slovaca*, then the sculpture pattern of the scape of *S. slovaca* is indicated as likely an intermediate stage in the development of the sculpture



Figs. 7–16. Figs. 7–12, outer and inner surface, respectively, of female scape: 7 and 8, *S. chontalensis*; 9 and 10, *S. slovaca*; 11 and 12, *S. nigroaenea*. Figs. 13–16, middle part of outer and inner surface, respectively of female scape: 13 and 14, *S. nigroaenea*; 15 and 16, *S. slovaca*.

pattern characteristic of female *S. nigroaenea.* The above-mentioned hypotheses of character transformation and relationships among *S. chontalensis*, *S. nigroaenea*, and *S. slovaca* can be tested by including samples of *S. chontalensis* in future evaluations of the D2-D3 region of the 28s ribosomal gene and the nuclear rDNA 18s gene. Furthermore, sculpture pattern of the scape seems to be a valuable feature for differentiating at least females of *Spalangia* species and should be investigated more comprehensively.

Bouček (1963) considered *S. cameroni* to have a secondarily worldwide distribution with the exception of North America and Australia. However, Burks (1969) subsequently synonymized *S. muscidarum* variety *texensis* Girault (type collected from Dallas, TX, in 1912) under *S. cameroni*, which indicates the species has been present in North America since at least 1912. Currently, *S. cameroni* is the most prevalent *Spalangia* species throughout most of the United States, especially the eastern United States (Rueda and Axtell 1985). *S. gemina* was imported from Brasil and reared in culture for at least 14 generations in Gainesville, FL (Geden 1996), but it was not released and is not known to occur in America north of Mexico. Genetic distance between *S. cameroni* and *S. gemina* supports the conclusion of Bouček (1963) that these two species are closely related. Bouček (1963) also keyed *Spalangia longepetiolata* Bouček with *S. cameroni* + *S. gemina*, a relationship that is supported by their similar pronotal sculpture. *S. longepetiolata* was introduced to

California from Kenya and Uganda in 1967 and 1968 and became established according to Legner (1978). As described above, both sexes of *S. cameroni* and *S. gemina* have a more or less vertical mesopleural anterior oblique impression (cf. Fig. 4), and both sexes lack setae from the forewing basal cell, whereas *S. longepetiolata* has a larger, more triangular anterior oblique impression (cf. Fig. 3) and males, although not females, have the forewing basal cell partly setose. Both sexes of *S. longepetiolata* also have a malar sulcus, which is lacking in *S. cameroni* and *S. gemina. S. gemina* is most readily differentiated from *S. cameroni* by relative length of the malar space and eye. The malar space is at least slightly longer than the length and distinctly longer the width of the eye in *S. cameroni*, whereas in *S. gemina* the malar space is distinctly shorter and only about the same length as the width of the eye. Both sexes of *S. gemina* also have shorter apical funicular segments than those of *S. cameroni*, being slightly transverse compared with quadrate or slightly longer than wide in females (Bouček 1963, cf. figs. 56, 24), and quadrate compared with oblong in males.

S. endius is a cosmopolitan species. Genetic distance between *S. endius* and *S. nigra* does not support the "*nigra*" and "*nigroaenea*" species-groups of Bouček (1963), because Bouček included *S. endius* in the *nigroaenea* rather than the *nigra*-group. However, several of the morphological features discussed above also do not support the monophyly of either the *nigra*- or *nigroaenea*-groups. Morphological evidence for relationships of *S. nigra* and *S. endius* is equivocal. *S. endius* shares a similar structure of the mesopleural anterior oblique impression with *S. nigra* (Fig. 3) and the other two *nigra*-group species not included in the molecular analysis, but also with *S. longepetiolata.* However, a conspicuously setose petiole and a more coarsely sculptured body indicate a closer relationship of *S. nigra* with the other two members of the *nigra*-group and with *S. nigroaenea* and related species within the *nigroaenea*-group.

Discussion

Specimens of *S. slovaca*, from Kazakhstan and Russia, were identified originally as European populations of *S. nigroaenea* until the molecular analyses indicated a species difference. This hypothesis was supported subsequently through morphological study, and the identification was revised after comparison of specimens with a paratype of *S. slovaca.* This species was described by Bouček (1963) based on two females from southeastern Slovakia, and the populations from Kazakhstan and Russia represent new distribution records.

The ITS-1 region was much more divergent among *Spalangia* spp. than *Muscidifurax* spp. Kimura two-parameter genetic distances among *Muscidifurax* spp. were ≤ 0.07, whereas numerous deletions/insertions made alignment of most of the ITS-1 region impossible among the *Spalangia* species. No differentiation among New and Old World populations of either *S. cameroni* or *S. endius* was observed. The two morphologically very similar species, *S. nigroaenea* and *S. slovaca,* were easily resolved in the ITS-1 region. The *S. nigroaenea* amplicon was 140 bp longer than that of *S. slovaca.* Multiple, independent insertion/deletion events were responsible for the size difference.

As with the ITS-1 amplicon, no diagnostic differences were observed in the 28s D2-D3 region among New and Old World samples of *S. cameroni* or *S. endius.* Samples of *S. nigroaenea* and *S. slovaca* differed by a genetic distance of 0.015. Interspecific genetic differences among the *Spalangia* species included in this study were 0.037–0.100, whereas that between *M. raptor* and *M. zaraptor* it was 0.004. Further samples from all biogeographic regions will be necessary to verify the species status of putatively cosmopolitan species and to test for unrecognized sibling species.

All of the *Spalangia* species examined in this study could be differentiated easily with restriction digests of the ITS-1 amplicon, which expands upon the number of species that can be differentiated using the technique of Ratcliffe et al. (2002) for identifying immature parasitoids inside the host puparium. Although the genetic distance between *S. nigroaenea* and *S. slovaca* was smaller than that observed between the other recognized *Spalangia* species included in this study, it was nearly 4 times greater than the genetic distance between *M. raptor* and *M. zaraptor.* The genetic distance between *S. nigroaenea* and *S. slovaca* supports their consideration as distinct species.

Numerous structural, sculptural, and setal features of *Spalangia* species are suitable for phylogenetic analysis; however, most are shared among the species in different combinations, which complicates hypotheses of polarity and resolution of relationships based on morphology. An accurate hypothesis of relationships based on morphology is not possible without a comprehensive study of the 51 valid world species listed by Noyes (2002). Although preliminary because of the small number of species included, our study clearly demonstrates that the D2-D3 region of the 28s ribosomal gene and the nuclear rDNA 18s gene provide an alternative method of phylogenetic analysis that also can be used to test or supplement hypotheses derived from morphology. Inclusion of additional species, such as *S. irregularis, S. rugulosa, S. chontalensis,* and *S. longepetiolata,* in future molecular studies should help resolve relationships among the species comprising the *nigra*- and *nigroaenea*-species groups. Such a method may prove effective for establishing a phylogenetic set of relationships among the species of *Spalangia* incrementally, rather than a single comprehensive study of the world species made necessary by morphology.

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