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A Cryptic Species Allied to *Halictus ligatus* Say (Hymenoptera: Halictidae) Detected by Allozyme Electrophoresis

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ABSTRACT: The social biology of *Halictus ligatus* has been investigated in many localities from Southern Canada to the Caribbean. In southern Florida it seems to be multivoltine and continuously brooded unlike the situation in more northerly areas where it has a more typical annual colony cycle with a moderately well established reproductive division of labor. In order to investigate the possibility of genetic differentiation between southern and northern behavioral types, samples of this species were collected along a transect from Toronto, Ontario in the north, to the Florida Keys in the South; additional samples were available from New Mexico and California. Two distinct species were found but surprisingly, their geographical ranges abut far to the north of the behavioral disjunction. The two species are differentiated by no fewer than 7 fixed differences out of 34 loci surveyed using standard gel electrophoretic techniques. It is probable that true *H. ligatus* is the northern form and that the Southeastern species should be called either *H. poeyi* Lepeletier or *H. capitosus* Smith. The two species are sympatric along a narrow stretch around the southern end of the Appalachian Mountains. Several biogeographic hypotheses are suggested which may account for the distribution of these two taxa. Further samples are required from the Southern USA, Central America and the Caribbean to differentiate these hypotheses. Comparisons of the social biology of these two species in an area of sympatry should be performed.

Halictus ligatus is a widespread and common social halictine that has been the subject of many field studies (reviewed by Michener and Bennett, 1977 and Packer and Knerer, 1986, see also Richards and Packer, 1995). This field work has encompassed much of the species' geographic range, which extends from southern Canada to Venezuela and across the North American continent. Packer and Knerer (1987) showed that the southern Florida populations were continuously brooded and multivoltine whereas those in the north of Florida had annual eusocial colony cycles more similar to those typical of other social halictines and other populations of *H. ligatus*. Populations in central Florida exhibited a phenological pattern that was not readily attributable to either type of colony cycle. Because of this behavioral differentiation and its potential role in effecting speciation (West Eberhard, 1986), an electrophoretic study was performed to determine if genetic differentiation paralleled the behavioral variation. In this paper we present a summary of the electrophoretic data that indicates that there are two species currently referred to as *Halictus ligatus*.

Materials and Methods

Collection of samples: The bees used in this study were obtained as follows. The Florida bees were collected along the length of the peninsula in April 1993. Samples of Ontario bees were obtained in June 1994 from the campus of York University, North York, Ontario. Samples from intermediate areas were collected from West Virginia south to Georgia in August 1994. Additional samples were

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Table 1. Sample sites and average sample sizes for the loci scored for *Halictus "ligatus"*.

Sample #	State/Province	Locality	Mean sample size per locus (SE)
1	New Mexico	Capilla Canyon	10.8 (1.0)
2	California	Yucaipa	14.6 (0.8)
3	Ontario	North York	15.9 (0.9)
4	West Virginia	Martinsburg	10.1 (0.3)
5	Virginia	Natural Bridge	10.9 (0.2)
6	Virginia	Fancy Gap	19.0 (2.3)
7	Virginia	Nettleridge	12.5 (0.7)
8	North Carolina	Statesville	13.4 (0.8)
9	North Carolina	Canton	19.1 (2.1)
10	Tennessee	Ocoee	15.1 (1.1)
11	North Carolina	Charlotte	12.1 (0.4)
12	Tennessee	Chattanooga	22.5 (2.0)
13	South Carolina	Rock Hill	12.9 (0.3)
14	Georgia	Macon	10.0 (0.3)
15	Georgia	Garden City	12.7 (0.6)
16	Florida	Lake City	16.4 (0.9)
17	Florida	Bee Line	5.8 (0.4)
18	Florida	Dade City	15.5 (0.8)
19	Florida	Bee Ridge	12.3 (0.7)
20	Florida	Fort Myers	14.5 (0.7)
21	Florida	Homestead	16.1 (0.8)
22	Florida	Marathon	15.9 (0.9)

obtained from Yucaipa, California and Cappilla Canyon, New Mexico, in August 1993. All bees were collected as they foraged at flowers. Table 1 lists all study sites and mean sample size per locus and Fig. 1 shows their location. The samples were stored in liquid nitrogen in the field, and thereafter in a −80°C freezer at York University.

Electrophoretic methods: Details concerning gel running conditions, enzyme staining recipes and scoring procedures used in this study can be obtained from earlier papers (Packer and Owen, 1989, 1990, 1992). For most analyses only the abdomen of the bees was used for electrophoresis. Because of the different sizes of the individuals sampled, bees were homogenized in varying amounts of grinding buffer (1% solution of dithiothreitol in double distilled water), ranging from 40 μl for the smallest, to 60 μl for the largest individuals.

Initially, runs were performed on subsamples from all collection sites, and these demonstrated a number of fixed differences between bees from the Southeast and the rest. Most of the loci exhibiting fixed differences were scored on two buffer systems (bI and bV). Consequently, to improve efficiency, we attempted to stain the remaining three loci (*Aat-1, Ak, Est-1*) also using these buffers (Table 2). Lists of loci scored, acronyms and associated enzyme commission numbers are given in Table 2, with the loci and buffer systems used in the optimized conditions being denoted with an asterisk.

Genetic distance and identity measures (Nei, 1978) were obtained using BIOSYS (Swofford and Selander, 1978). Estimates were obtained in a hierarchical manner using the Step Hierarchy algorithm in BIOSYS with two different schemes, first between the two genetically differentiated forms and among pop-

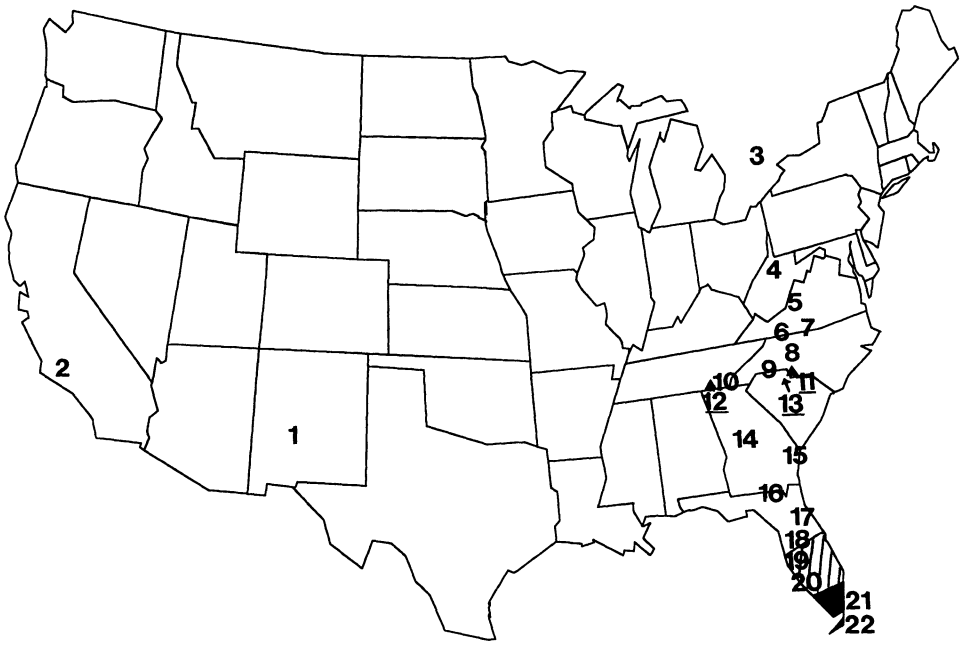

Fig. 1. Map of the United States of America showing the location of sampling sites and areas of behavioral differentiation as described in the text. The shaded area in southern Florida indicates where continuously brooded, multivoltine populations occur, the stippled area represents the area where phenologies were ambiguous, in all other areas in North America annual colony cycles occur. The underlined areas represent sites where the two species were sympatric. Because collections were made at many nearby localities, the numbers referring to Charlotte (11) and Chattanooga (12) had to be placed somewhat away from their precise position on the map. A closer approximation to these locations is indicated by the filled triangle symbols. The precise location of Rock Hill (13) is indicated with an arrow.

ulations within each form, and then between the continuously brooded, multivoltine populations in Homestead, FL and Marathon, FL versus the remaining Southeastern form genotypes from areas where an annual phenology is expected (Packer and Knerer, 1987), i.e., Dade City, Bee Line and Lake City in Florida and Macon and Garden City in Georgia.

Electrophoretic comparisons were made between the genetically differentiated form and some of the other species of *Halictus* used in the phylogenetic study of Richards (1994), namely *Halictus confusus, H. maculatus* and *H. scabiosae.*

Results

There were 7 fixed differences between Southeastern samples and those from the rest of North America; at the loci *Aat-1, Ak, Est-1, G3pdh-2 Gpi, Idth* and *Me.* Table 3 lists the relative mobilities and geographic distribution of alleles at these loci (a full account of variation within the two species, heterozygosity, and gene flow will be published elsewhere, pending further sampling). The two forms were found sympatrically at three locations: Chattanooga, Tennessee; Rock Hill, South Carolina and Charlotte, North Carolina (Fig. 1). The number of individuals of each form from these three areas is provided in Table 4. In each case all females

Table 2. Enzyme names, abbreviations, EC numbers and buffer systems used.

Enzyme	Acronym	EC number[a]	Buffer[b]
Aspartate aminotransferase*	*Aat-1 & 2*	2.6.1.1	RSL, V*
Acid phosphatase	*Acp*	3.1.3.2	I
Aminoacylase	*Acy*	3.5.1.14	RSL
B-N-Acetylhexosaminidase	*Aha*	3.2.15.2	I
Adenylate kinase*	*Ak*	2.7.4.3	CAM, I*
Aldehyde dehydrogenase	*Alddh*	4.1.2.13	V
Arginine kinase	*Ark*	2.7.3.3	I
Diaphorase (NADH)	*Dia*	1.8.1.n	V
Diaphorase (NADPH)	*Diaph*	1.9.99.n	V
Esterase*	*Est-1 & 2*	3.1.1.1	RSL, V*
Fumarate hydratase	*Fum*	4.2.1.2	I
B-Galactosidase	*BGal*	3.2.1.23	I
Glycerol-3-phosphate dehydrogenase*	*G3pdh-1 & 2*	1.1.1.8	I*
Glucose-6-phosphate isomerase*	*Gpi*	5.3.1.9	I*
Glucose-6-phosphate dehydrogenase	*G6pdh*	1.1.1.49	CAM
B-Hydroxyacid dehydrogenase	*Had*	1.1.1.30	I
Hydroxyacylglutathione hydrolase	*Hagh*	3.1.2.6	V
Hexokinase	*Hk*	2.7.1.1	I
L-Iditol dehydrogenase*	*Idth*	1.1.1.15	V*
Malate dehydrogenase	*Mdh-1 & 2*	1.1.1.37	CAM
Malate dehydrogenase NADP*	*Me*	1.1.1.40	I*
Peptidase glycine-leucine	*Pepgl*	3.4.11.n	RSL
Peptidase leucine-alanine	*Pepla*	3.4.11.13	RSL
Peptidase phenylalanine-proline	*Peppp*	3.4.13.8	RSL
Phosphogluconate dehydrogenase	*6Pgd*	1.1.1.43	CAM
Phosphoglucomutase	*Pgm*	5.4.2.2	I
Pyruvate kinase	*Pk*	2.7.1.40	V
Superoxide dismutase	*Sod*	1.15.11	CAM
Triosephosphate isomerase	*Tpi*	5.3.1.1	RSL
Uridine D-glucose pyrophosphorylase	*Ugpp*	2.7.7.9	RSL

[a] EC numbers taken from Webb (1984).

[b] Buffers I and V taken from Shaw and Prasad (1970), RSL from Ridgway et al. (1970) and CAM from Clayton and Tretiak (1972).

* Indicates enzymes which showed fixed differences between species and the buffer systems that were used for these loci in the reoptimized conditions (see text for details).

were homozygous for the entire suite of Southeastern or the Northern and Western alleles; the two males had the entire suite of Northern alleles. Based upon these unambiguous results, we conclude that *Halictus ligatus* is composed of two species, one apparently restricted to the Southeastern U.S.A.

Nei's (1978) genetic identity between the species was low; I = 0.709, whereas that among populations within each species was high (0.946 to 0.963, Table 5). According to the calibrations of Brussard et al., (1985), the genetic identity values are consistent with species level differentiation between the two forms and local population differentiation among populations within them.

The genetic disjunction did not occur across the area of behavioral differentiation. Genetic differentiation among populations on either side of this area was minimal with a hierarchical identity value of 0.969 between behavioral forms (Table 6). This was less than the value between the two continuously brooded

Table 3. Mobilities for alleles of the 7 loci exhibiting fixed differences between species and their geographic distribution.

Locus	Mobility (mm)[a]	Sample localities[b]
Aat-1	−10	11–22
	+1	1–13
Ak	10	11–22
	22	1–13
Est-1	32	11–22
	38	1–13
G3pdh-2	25	1–13
	36	11–22
Gpi	−10	1–13
	+1	11–22
Idth	21	1–13
	23	11–22
Me	26	1–13
	32	11–22

[a] Mobilities are given in millimetres rather than as relative to a standard of 100 for the most common allele because i) fixed differences between species are being referred to here and ii) two loci would require mobilities of 100 and −1000 if the relative to the common species standard were to be used.
[b] Locality numbers refer to sample sites listed in Table 1, but as can be seen, all diagnostic loci were found only allopatrically except for sites 11–13 where both species were sympatric.

samples (0.998) but slightly larger than that among the annual samples of the Southeastern form (0.951). This suggests that there is no barrier to gene flow between behavioral types in Florida.

None of the electromorphs that were diagnostic for the new species provided synapomorphies with any of the other species of *Halictus* surveyed.

Discussion

We have detected a cryptic species allied to *Halictus ligatus* using gel electrophoretic techniques. The two taxa are separated by 7 fixed differences and occur in sympatry along the southern margin of the Appalachian Mountains with no evidence of gene flow for these 7 loci between the two species even in areas where they are sympatric. In contrast, there was no clear pattern to genetic differentiation within each species even though the California and New Mexico populations were thousands of kilometers distant from the Eastern samples.

Halictus ligatus was originally described by Say in 1837. Although the type

Table 4. Number of haploid genomes of the two species caught in areas where the two are sympatric. In each case every individual contained only the alleles diagnostic for its species at all 7 loci exhibiting fixed differences between species.

Locality	*Halictus ligatus*	*Halictus poeyi/capitosus*
Chattanooga, TN	17*	2
Charlotte, NC	7*	4
Rock Hill, SC	12	16

* One male contributed to each of these two samples, all other individuals were female.

Table 5. Genetic identity values (Nei, 1978) among populations and between species.

Species	Number of populations	Genetic identity, mean (range)	
		ligatus	*poeyi/capitosus*
ligatus	13	0.963 (0.922–1.000)	
poeyi/capitosus	12	0.709 (0.620–0.763)	0.946 (0.875–0.998)

has been lost it seems safe to assume that it was not obtained from the Southeastern U.S.A. Sandhouse (1941) lists the synonyms for *H. ligatus* as understood hitherto and also provides type locality data where known. It seems likely that the newly differentiated species should be referred to as either *Halictus poeyi* Lepeletier or *H. capitosus* Smith. The type locality for the former is Cuba, the latter is from Florida. If both names are attributable to the Southeastern species then the name *H. poeyi* would take precedence.

At each site where both species were collected they were found flying together in small areas. For instance, at Charlotte the sample was obtained along the top of a roadside embankment outside a motel where most of the bees were collecting pollen from *Angelica.* Clearly this is an example of real sympatry without small local scale habitat separation, although nest sites remain to be located for either species in an area of sympatry and there may be some habitat differentiation in nest site choice.

Three hypotheses may be suggested to explain the distribution of the two species under consideration: i) *H. poeyi/capitosus* became differentiated in situ in Florida, ii) it is of Central American origin and migrated to the Southeastern states around the Gulf of Mexico and iii) it is of Central American origin but has migrated to Florida via the Yucatan and Jamaica. Although additional sampling is required to differentiate these hypotheses, some preliminary discussion of them is provided here.

i) A wide range of taxa show disjunct phylogeographic distributions in the Southeastern U.S.A. For example, in their study of white-tailed deer, Ellsworth et al. (1994) found three distinct mtDNA haplotype lineages in this area. One was restricted to the Florida keys and Everglades, the second was found throughout the rest of Florida and in Georgia and South Carolina, and the third lineage was found further north and to the west of the Apalachicola river system. The first of these areas is identical to the area inhabited by apparently continuously brooded

Table 6. Genetic identity measures (Nei, 1978) within and among populations either side of the behavioral disjunction in southern Florida for *Halictus poeyi/capitosus.*

Behavioral type	Number of samples	Genetic identity, mean (range)	
		Continuous	Annual
Continuously brooded, multivoltine	2	0.998	(−)
Annual, univoltine	5	0.969 (0.930–0.994)	0.951 (0.911–0.986)

and multivoltine populations of *"Halictus ligatus,"* although we have found no evidence of an interruption of nuclear gene flow between bees from this area and the rest of the Southeast. The boundary between the remaining two white-tailed deer mtDNA haplotype lineages coincides with the disjunction between *Halictus ligatus* and *H. poeyi/capitosus.* Ellsworth et al. (1994) describe other taxa which, based upon mtDNA, are differentiated across the same boundary; these include bowfin *Amia calva,* woodrats *Neotoma floridana,* and pocket gophers *Geomys pinetis.* They suggest that differentiation could have occurred during the Pleistocene as a result of sea level fluctuations which would have cut off the Ocala highlands area in central Florida, where differentiation is believed to have occurred, from land masses further north. It is possible that the same geological events led to the separation of the two species of *Halictus* with the Appalachian mountain chain forming a barrier preventing further northward dispersal of the peninsular isolate after sea levels subsided.

ii) *Halictus poeyi/capitosus* may have moved into the Southeastern U.S.A. along the coast of the Gulf of Mexico. It is believed that the Florida panther originated in this manner, as haplotypes of this form appear derived from South American ones (O'Brien et al., 1990). That several bee species found in the region of the Florida panhandle have their closest relatives in the deserts of the Southwestern U.S.A. (Cane, personal communication) suggests that other groups of bees may have followed this dispersal route.

iii) An alternative hypothesis is that *H. poeyi/capitosus* dispersed into this area from Central America via the Yucatan peninsula and Jamaica. Eickwort (1988) suggested this pathway for at least two species groups of *Dialictus* while noting that migration of sweat bees into the Carribean from Meso-America appears far more common than via the Lesser Antilles. That *H. poeyi/capitosus* is unlikely to have followed the Lesser Antillean route to the colonization of the larger islands and thereby Florida and the Southeastern states is suggested by its absence from Hispaniola, Puerto Rico and the Lesser Antilles (Eickwort, 1988) as well as its apparently recent colonization of South America (Michener and Bennett, 1977). It is possibile that *Halictus ligatus* and its sister species form a ring complex around the Carribean with gradual change from northern to Southeastern genetic types through Mexico, Central America and Cuba, with the two extremes meeting in the southern Appalachians.

Richards (1994) performed a phylogenetic analysis of some species in the genus *Halictus* which gave two clusters of most parsimonious trees of very different topology. One set had *H. ligatus* (sampled from Ontario) occupying a basal position, the other had *H. rubicundus* as sister taxon to the remaining species. The latter is in closer agreement to morphological analyses (Pesenko, 1985). It seemed possible that the electromorphs which were diagnostic for the new species might provide some synapomorphies for the *H. ligatus* clade and other taxa included in Richards' study. However, none of the 7 loci showed synapomorphic variation between the new species and the three other species of *Halictus* surveyed here.

We would like to comment on the unexpected nature of this discovery. *Halictus ligatus* is one of the most abundant and readily identifiable bees in North America. It certainly is known as a variable species, but this is not surprising given its large geographic range, encompassing a wide range of aridities and altitudes, as well as the additional complexities caused by allometric variation and size-based

caste differentiation. Indeed, morphological variation in *H. ligatus* has been the subject of a Ph.D. thesis (Kirkton, 1968) and no obvious disjunction was detected. Although other species groups in the Halictini are well known as being taxonomically "difficult", there has never been any doubt that *Halictus ligatus* was a "good" species. It seems probable that such ease of identification has discouraged the critical examination which taxa that are "known" to be "difficult" receive. This situation appears to be not uncommon in bees (Packer and Taylor, submitted) and warrants investigation in other taxa.

The original impetus for this study was the investigation of genetic variation between behavioral types. We found no evidence for any genetic differentiation across the behavioral boundary in Central Florida, where populations appear to be panmictic with high genetic identities. However, we did find evidence of species level differentiation much further north. We now need to investigate whether behavioral or phenological differences exist between the two species in areas of sympatry.

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NOTE ADDED IN PROOF:

Since the analyses discussed here were performed, additional sampling has demonstrated that the "southeastern" form occurs further north than noted here. A small sample of the Southeastern species was obtained at Weldon, on the coastal plains region of North Carolina just South of the Virginia border. The Southeastern species probably occurs even further North along the Eastern seaboard making it possible that the name *H. ligatus* applies to this species rather than the Northern one.