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Allozyme variation and differentiation among four *Apis* species

W.S. Sheppard [1] and S.H. Berlocher [2]

[1] *USDA-ARS, Bldg. Beneficial Insects Laboratory, 476, Beltsville, MD 20705, USA*
[2] *University of Illinois, Department of Entomology, Urbana, IL 61801, USA*

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Summary — An electrophoretic survey of 18 putative enzyme loci was conducted on Sri Lankan *Apis cerana*, *A. dorsata* and *A. florea*, using techniques of sequential electrophoresis. Included for analysis were published data from European *A. mellifera*. Seven enzymes exhibited polymorphism and for some species are reported here for the first time. Seven enzymes showed fixed allozymic differences among all four species and three were fixed for the same allozyme in all four species. Allozyme data were analyzed using UPGMA clustering, a Distance Wagner procedure and a simple cladistic method. The mean genetic distance (Nei's $D = 1.30$) indicates that considerable allozyme differences have accumulated among the species. Due to the relatively large genetic distance between *A. cerana* and *A. mellifera* ($D = 1.10$), electrophoretic data do not support a divergence time for these species as recent as that suggested by morphological and behavioral evidence. The electrophoretic data do not resolve questions concerning the phylogeny of *Apis*, due largely to the number of fixed differences among the species. Electrophoresis does show promise for species identification and resolution of the *A. dorsata* "complex" and other issues in *Apis*, as even the entry of data from single colonies into the BIOSYS program provided unambiguous discrimination. The treatment of electrophoretic data with phylogenetic tree-building or clustering techniques has potential for subspecies identification in endemic *A. mellifera*.

Apis **— phylogeny — species differentiation — biochemical polymorphism — allozyme**

INTRODUCTION

The honey bee genus *Apis* is best known for four species widely recognized by melittologists. Two of these, *A. mellifera* and *A. cerana,* the "Western" and "Eastern" honey bees, respectively, are cavity-nesting species that originally occurred only in the Old World tropics and temperate zones. The other two species, *A. dorsata,* the giant or rock bee, and *A. florea,* the dwarf honey bee, are both found in exposed single-comb nests from India through Southeast Asia and Indonesia.

Maa's (1953) systematic revision that split this group into three genera (after Ashmead 1904) and 24 species has not been widely followed, although Sakagami *et al.* (1980) have proposed that Maa's division of *A. dorsata* (*Megapis*) into four distinct species be recognized. In contrast to these proposals for subdivision of the genus, it has also been suggested that the two cavity-nesting species *A. mellifera* and *A. cerana* be consolidated into a single species, based on morphological, behavioral, or cytogenetic similarities (Kerr & Laidlaw, 1956; Deodikar *et al.,* 1959;

Deodikar *et al.*, 1961; Wilson, 1971). Uncertainty regarding the specific status of *A. mellifera* and *A. cerana* still appears in the literature (Culliney, 1983), although Ruttner and Maul (1983) conclusively demonstrated the presence of complete reproductive isolation between the species.

In addition to taxonomic questions about the status of particular species within *Apis*, the phylogenetic relationships among the members of the genus are a matter of some controversy. One of the well-known evolutionary schemes (Michener, 1974) is that an ancestral species of *Apis* gave rise to two phyletic lines, one leading to *A. florea* and the other to the three other species. The latter line then gave rise to the *A. dorsata* line and a line leading to the two cavity-nesters *A. mellifera* and *A. cerana*. *A. cerana* has been proposed to have given rise to *A. mellifera* somewhere in the Himalayas (Deodikar *et al.*, 1959). Alternatively, Koeniger (1976) proposed that cavity nesting is ancestral, with the aerial single combs of *A. florea* and *A. dorsata* arising as adaptations to tropical climates.

To-date, most discussion of the taxonomy and phylogeny of *Apis* has been based on behavioral, morphological, or cytogenetic evidence (Deodikar *et al.*, 1959; Kerr, 1969; Wilson, 1971; Michener, 1974; Koeniger, 1976; Ruttner, 1988), but has not considered data derived from molecular techniques. At the generic level, gel electrophoresis of enzymes is the molecular technique most widely applied to insect systematics (Berlocher, 1984). However, with the exception of a single species, *A. mellifera*, there exists very little published electrophoretic data for the genus. Thus, while a number of investigators have reported electrophoretic variation within *A. mellifera* (Mestriner, 1969; Sylvester, 1976; Pamilo *et al.*, 1978; Nunamaker, 1980; Sheppard and Berlocher, 1984, 1985; Sheppard and McPheron, 1986) there exists only a limited comparison of two enzymes (malate dehydrogenase and esterase) in three eastern *Apis* species (Nunamaker *et al.*, 1984; Li *et al.*, 1986), and a preliminary report of enzyme polymorphism for these species (Sheppard, 1985).

The purpose of the present study was to compare the four best-known *Apis* species electrophoretically and to bring the data to bear on the questions of the phylogenetic relationships and amount of differentiation among the species.

MATERIAL AND ELECTROPHORESIS

Samples of adult workers from ten colonies of *A. cerana*, five colonies of *A. dorsata* and ten colonies of *A. florea* were taken from locations throughout Sri Lanka (Table I). Data for *A. mellifera* were taken from previous studies on 23 colonies (representing three races : *A. m. mellifera*, *A. m. ligustica* and *A. m. carnica*) from Europe (Sheppard and Berlocher 1984, 1985; Sheppard and McPheron, 1986). The Sri Lankan samples were killed by freezing and were then placed in liquid nitrogen. Upon arrival at the laboratory, samples were stored at –70 °C. Samples were prepared by homogenizing head-thorax sections in a grinding buffer (Sheppard and Berlocher, 1984) and electrophoresing in horizontal slab starch gels. A preliminary "sequential" (Coyne, 1982) electrophoretic survey using five different gel/buffer conditions (Sheppard and Berlocher, 1985) was conducted, followed by single condition electrophoresis using the optimal buffer/gel condition for each enzyme. At least 15 workers per colony were examined for each of 15 enzymes. These were (including our abbreviation and Enzyme Commission number in parentheses): aconitase (ACON; 4.2.1.2), aldolase (ALDO; 4.1.2.13), alpha-glycerophosphate dehydrogenase (α-GDH; 1.1.1.8), arginine kinase (ARGK; 3.3.8.9), beta-hydroxybutyric acid dehydrogenase (β-HBDH; 1.1.1.30), esterase (EST; 3.1.1.1), glyceraldehyde-3-phosphate dehydrog-

enase (G-3-PDH; 1.2.1.12), hexokinase (HEX; 2.7.1.1), isocitrate dehydrogenase (IDH; 1.1.1.42), leucine aminopeptidase (LAP; 3.4.1.1), malate dehydrogenase (MDH; 1.1.1.37), malic enzyme (ME; 1.1.1.40), phosphoglucomutase (PGM; 2.7.5.1), phosphoglucose isomerase (PGI; 5.3.1.9) and triose phosphate isomerase (TPI; 5.3.1.1). Two additional enzymes, aldehyde oxidase (AO; 1.2.3.1) and an NAD-dependent isocitrate dehydrogenase (IDHN; 1.1.1.41) were stained during the preliminary survey and in many of the later runs. Both enzymes were invariant within species for all individuals tested and were included in the analysis. Enzyme activity was visualized with standard histochemical staining techniques (Sheppard and Berlocher, 1984, 1985) and gels were photographed for documentation. All four species were run in side-by-side lanes for comparison of relative mobilities and allozyme congruence in the initial sequential survey. Allozyme designations are based on relative mobilities, with decimal points omitted. For MDH, the "fast" allozyme of *A. mellifera* (Mdh^{100}; Sheppard and Berlocher, 1984) is used as a standard (mobility 1.00). For all other enzymes, the most common allozyme of *A. mellifera* is used as a standard. Although genetic crosses to establish the Mendelian inheritance of *Apis* allozymes have only been performed on *Mdh* and *Est* in *A.mellifera* (Mestriner and Contel, 1972; Contel *et al.*, 1977; Bitondi and Mestriner, 1983), all the enzyme banding patterns were consistent with a simple genetic basis for the variation.

Analyses of electrophoretic data

Measures of genetic variation at enzyme loci are fairly standardized (heterozygosity, number of alleles per locus, percent loci polymorphic). By contrast, a number of methods can be used to construct phylogenetic trees from electrophoretic data (Felsenstein, 1982; Berlocher, 1984). For this study we used three : 1) the widely-used technique of UPGMA clustering based on Nei distances, 2) a cladistic analysis of the phylogeny of the species, and 3) a Distance Wagner analysis based on a genetic distance matrix. These methods have been discussed, along with some of their advantages and disadvantages, by Felsenstein (1982) and Berlocher (1984). Briefly, the UPGMA method produces a tree from a matrix of pair-wise distances between the taxa. The two taxa separated by the smallest genetic distance are joined and a third taxon, the one with the smallest mean distance to the previously united taxa, is added. The process of adding taxa is continued in this manner until all of the taxa are included. One assumption inherent in UPGMA clustering is that the evolutionary rate in all lineages is equal, a limitation that was recognized by its early proponents (Michener and Sokal, 1957). A similar assumption is involved in the distance measure used in the UPGMA. Nei's genetic distance (Nei, 1971) assumes that evolutionary rates among enzyme loci are equal and, since it is based on a neutral-mutation model, that evolution of proteins occurs in a "clock-like" fashion (Kimura, 1983). When performing the UPGMA clustering, only 15 of the 23 colonies of *A. mellifera* were included in the analysis. With the exception of a single colony (from a site where two had been sampled) of Norwegian *A. m. mellifera*, data from all previously analyzed colonies of *A. m. mellifera* (5) and *A. m. ligustica* (5) were used (Sheppard and Berlocher 1984, 1985). Of the Czechoslovakian bees, we included the five colonies considered most typical of *A. m. carnica*, based on cubital index measurements (Table 4.2 of Sheppard and McPheron, 1986), since Czechoslovakia constitutes a zone of hybridization between *A. m. mellifera* and *A. m. carnica* (F. Ruttner, personal communication).

Although conventional cladistic analyses of allozyme data are incapable of dealing with frequency data (Berlocher, 1984), we were able to perform a simple cladistic analysis on the species of *Apis* because all phylogenetically informative loci were monomorphic (see Results). However, the races of *A. mellifera* differ at polymorphic loci, preventing a straight cladistic analysis. As an alternative to UPGMA, we performed a Distance Wagner analysis of the matrix of Prevosti genetic distances (Berlocher, 1984). The Distance Wagner analysis makes fewer assumptions about the way evolution proceeds than does UPGMA in that no molecular clock is implied, nor is an equal rate of evolution required for each lineage. The UPGMA and Distance Wagner analyses were performed using the BIOSYS computer program for electrophoretic data by Swofford and Selander (1981).

RESULTS

Extent of variation in honey bees

Seven of the enzymes exhibited polymorphism in the four honey bee species. The distribution of these polymorphisms among the species, along with the number of allozymes per polymorphic enzyme (in parentheses) was as follows: *A. cerana* was polymorphic for four of the enzymes — ACON (2), EST (2), MDH (2) and ME (2); *A. dorsata* showed variation in one — HEX (2); *A. florea* exhibited polymorphism for three — ME (2), PGM (3) and TPI (2); and five polymorphic enzymes have been reported in European *A. mellifera* — ACON (2), EST (3), MDH (5), ME (3) and PGM (2) (Sheppard and Berlocher, 1984, 1985; Sheppard and McPheron, 1986). With the exception of aconitase, only a single locus was scored for each enzyme. As in *A. mellifera* (Sheppard and McPheron, 1986), aconitase in the other *Apis* species exhibits two putative loci, *Acon-1* (cathodally migrating isozymes) and *Acon-2* (anodally migrating isozymes). In the two species exhibiting aconitase polymorphism, *A. mellifera* and *A. cerana,* variation was present only in the anodally migrating (Acon-2) allozymes. Allozyme frequencies of the polymorphic enzymes are reported in Table I. The single EST system consistently scorable for all *Apis* species under our conditions appears to be encoded by the locus designated *Est-3* by Bitondi and Mestriner (1983; see discussion in Sheppard and Berlocher, 1985). The subunit structure of the polymorphic enzymes, suggested by the banding pattern of heterozygotes, was constant across all species in which they occurred. These structures have previously been reported for the five polymorphic enzymes of adult *A. mellifera* (Mestriner, 1969; Mestriner and Contel, 1972; Sheppard and Berlocher, 1984; Del Lama *et al.,* 1985; Sheppard and McPheron, 1986). Triose phosphate isomerase, polymorphic only in *A. florea,* exhibited three bands in heterozygous individuals indicating a dimeric molecule. Human TPI has also been suggested to be dimeric (Harris and Hopkinson, 1976). Hexokinase, polymorphic only in *A. dorsata,* displayed two-banded heterozygotes which suggests a monomeric structure. Monomeric HEX is also found in human subjects (Harris and Hopkinson, 1976).

Seven enzymes showed fixed allozymic differences among all four honey bee species (G-3-PDH, EST, HEX, AO, IDH, ME, PGM). Three enzymes were fixed for the same allozyme in all four species (ß-HBDH, LAP, PGI). Each of the remaining eight enzymes had allozymes that were shared between species. The allozyme distribution among species for monomorphic enzymes is shown in Table II.

The UPGMA analysis of Nei unbiased genetic distances for forty colonies is shown in Fig. 1. The UPGMA analysis clustered all conspecific colonies together, so for simplicity the Figure is drawn showing the pooled result. Figure 2 shows the cladistic analysis based on the three phy-

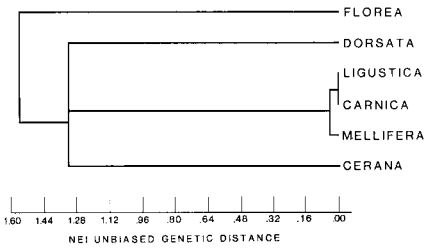

Fig. 1. UPGMA clustering analysis of four species of *Apis* based on Nei unbiased genetic distance. FLOREA = *A. florea;* DORSATA = *A. dorsata;* CERANA = *A. cerana;* LIGUSTICA = *A. mellifera ligustica;* CARNICA = *A. m. carnica* and MELLIFERA = *A. m. mellifera.*

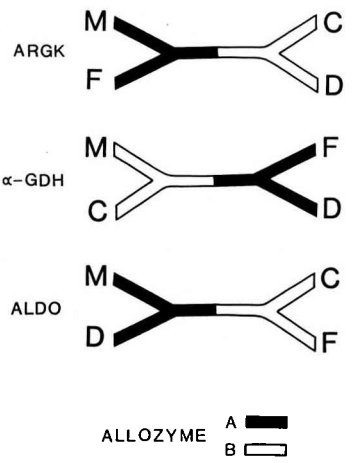

Fig. 2. The three possible unrooted trees for *Apis*, showing allozyme characters supporting each. Shared letter denotes shared allozymes. Species abbreviations: M = *A. mellifera*, C = *A. cerana*, D = *A. dorsata*, F = *A. florea*.

logenetically informative enzymes (see Discussion). Finally, Fig. 3 shows the results of the Distance Wagner procedure on individual colonies of *A. mellifera* with the tree rooted at the midpoint.

DISCUSSION

An obvious conclusion that can be drawn from the electrophoretic data is that the four species are quite divergent from one another. That is, not only are these "good" species, but they must be fairly old species. This is evidenced by the accumulation of fixed differences among the species for seven enzyme loci and the considerable mean genetic distance for the four species: D = 1.30. For comparison, morphologically distinct species of the *Drosophila*

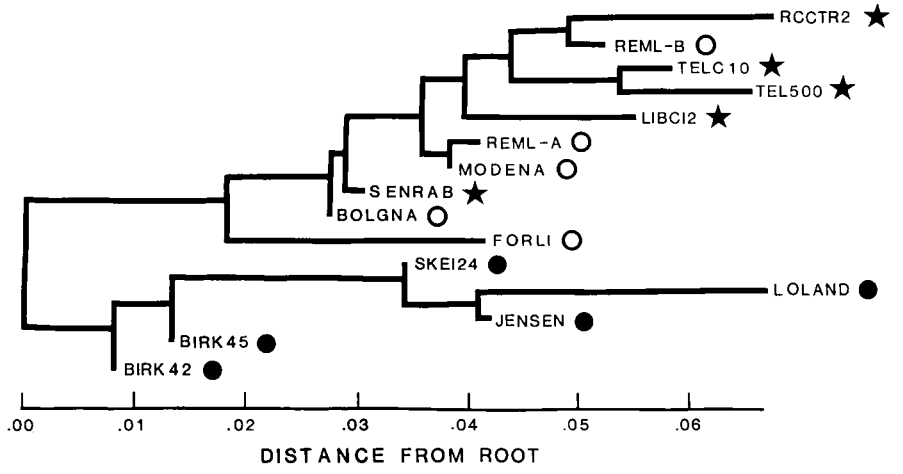

Fig. 3. Distance Wagner analysis (based on Prevosti distances) of three races of *Apis mellifera* with midpoint rooting. Key to races: *A. m. mellifera* (●); *A. m. ligustica* (o); *A. m. carnica* (*). Key to locations: *A. m. mellifera* (Norway): JENSEN (Jensen); SKEI24 (Skeibrok); BIRK42, BIRK45 (Birkeland). *A. m. ligustica* (Italy): REML-A, REML-B (Reggio Emilia), MODENA (Modena), BOLOGNA (Bologna), FORLI (Forli). *A. m. carnica* (Czechoslovakia): RCCTR2 (Ricany Center), TEL500, TELC10 (Telc), LIBCI2 (Libcice), SENRAB (Senohraby).

Table I. Colony allozyme frequencies of polymorphic enzymes in *Apis*.

Collecting locality, Sri Lanka	*Enzyme*		
	MDH	*Est*	*ME*
(Apis cerana)			
1. Weliara	Mdh^{109}=1.00	Est^{57}=0.97 Est^{86}=0.03	Me^{91}=1.00
2. Wallawaya	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
3. Hingurukaduwa	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
4. Thellula	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
5. Thanamalwila	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
6. Moneragala	Mdh^{109}=0.97 Mdh^{75}=0.03	Est^{57}=0.97 Est^{86}=0.03	Me^{91}=1.00
7. Athimale	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
8. Madagama	Mdh^{109}=0.95 Mdh^{75}=0.05	Est^{57}=1.00	Me^{91}=1.00
9. Yudagannawa	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=1.00
10. Bibile	Mdh^{109}=1.00	Est^{57}=1.00	Me^{91}=0.97 Me^{110}=0.03
(Apis dorsata)			
11. Wallawaya	Mdh^{109}=1.00	Est^{134}=1.00	Me^{86}=1.00
12. Athimale	Mdh^{109}=1.00	Est^{134}=1.00	Me^{86}=1.00
13. Kumbukkana	Mdh^{109}=1.00	Est^{134}=1.00	Me^{86}=1.00
14. Madagama	Mdh^{109}=1.00	Est^{134}=1.00	Me^{86}=1.00
15. Balaharuwa	Mdh^{109}=1.00	Est^{134}=1.00	Me^{86}=1.00
(Apis florea)			
16. Bodagama	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=1.00
17. Thellula	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=1.00
18. Kitulkote	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=0.89 Me^{76}=0.11
19. Anapallama	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=1.00
20. Petiyanara	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=1.00
21. Puhulkotuwa	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=0.90 Me^{76}=0.10
22. Madagama	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=0.87 Me^{76}=0.13
23. Mahagodayaya	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=0.93 Me^{76}=0.07
24. Okkampitiya	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=0.95 Me^{76}=0.05
25. Gangeyaya	Mdh^{71}=1.00	Est^{103}=1.00	Me^{84}=1.00
26 -(Apis mellifera) 40. Europe*	Mdh^{55}<0.01 Mdh^{65}=0.40 Mdh^{80}=0.33 Mdh^{87}=0.02 Mdh^{100}=0.25	Est^{70}=0.04 Est^{100}=0.92 Est^{130}=0.04	Me^{79}=0.06 Me^{100}=0.92 Me^{106}=0.02

ACON-2	PGM	HEX	TPI
$Acon2^{100}$=0.97 $Acon2^{114}$=0.03	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{100}$=1.00	Pgm^{90}=1.00	Hex^{119}=1.00	Tpi^{69}=1.00
$Acon2^{108}$=1.00	Pgm^{106}=1.00	Hex^{86}=1.00	Tpi^{100}=1.00
$Acon2^{108}$=1.00	Pgm^{106}=1.00	Hex^{86}=1.00	Tpi^{100}=1.00
$Acon2^{108}$=1.00	Pgm^{106}=1.00	Hex^{86}=1.00	Tpi^{100}=1.00
$Acon2^{108}$=1.00	Pgm^{106}=1.00	Hex^{86}=0.85 Hex^{91}=0.15	Tpi^{100}=1.00
$Acon2^{108}$=1.00	Pgm^{106}=1.00	Hex^{86}=1.00	Tpi^{100}=1.00
$Acon2^{88}$=1.00	Pgm^{112}=1.00	Hex^{103}=1.00	Tpi^{62}=0.93 Tpi^{27}=0.07
$Acon2^{88}$=1.00	Pgm^{112}=0.95 Pgm^{128}=0.05	Hex^{103}=1.00	Tpi^{62}=0.90 Tpi^{27}=0.10
$Acon2^{88}$=1.00	Pgm^{112}=1.00	Hex^{103}=1.00	Tpi^{62}=0.70 Tpi^{27}=0.30
$Acon2^{88}$=1.00	Pgm^{112}=1.00	Hex^{103}=1.00	Tpi^{62}=0.79 Tpi^{27}=0.21
$Acon2^{88}$=1.00	Pgm^{112}=0.97 Pgm^{128}=0.03	Hex^{103}=1.00	Tpi^{62}=1.00
$Acon2^{88}$=1.00	Pgm^{112}=1.00	Hex^{103}=1.00	Tpi^{62}=0.70 Tpi^{27}=0.30
$Acon2^{88}$=1.00	Pgm^{112}=0.92 Pgm^{103}=0.08	Hex^{103}=1.00	Tpi^{62}=0.80 Tpi^{27}=0.20
$Acon2^{88}$=1.00	Pgm^{112}=0.97 Pgm^{103}=0.03	Hex^{103}=1.00	Tpi^{62}=0.83 Tpi^{27}=0.17
$Acon2^{88}$=1.00	Pgm^{112}=0.97 Pgm^{103}=0.03	Hex^{103}=1.00	Tpi^{62}=0.87 Tpi^{27}=0.13
$Acon2^{88}$=1.00	Pgm^{112}=0.97 Pgm^{103}=0.03	Hex^{103}=1.00	Tpi^{62}=0.87 Tpi^{27}=0.13
$Acon2^{100}$=1.00 $Acon2^{120}$$<$0.01	Pgm^{100}=1.00 $Pgm^{75}$$<$0.01	Hex^{100}=1.00	Tpi^{100}=1.00

* Mean frequencies of 15 colonies; from Sheppard and Berlocher 1984, 1985; Sheppard and McPheron, 1986.

Table II. Allozyme distribution among *Apis* species (monomorphic enzymes).

	A. mellifera	*A. cerana*	*A. dorsata*	*A. florea*
ACON-1	Acon-1^{100}	Acon-1^{111}	Acon-1^{89}	Acon-^{189}
ALDO	Aldo^{100}	Aldo^{164}	Aldo^{100}	Aldo^{164}
α-GDH	α-Gdh^{100}	α-Gdh^{100}	α-Gdh^{74}	α-Gdh^{74}
AO	Ao^{100}	Ao^{75}	Ao^{115}	Ao^{111}
ARGK	Argk^{100}	Argk^{116}	Argk^{116}	Argk^{100}
β-HBDH	β-Hbdh^{100}	β-Hbdh^{100}	β-Hbdh^{100}	β-Hbdh^{100}
IDH	Idh^{100}	Idh^{92}	Idh^{67}	Idh^{90}
IDHN	Idhn^{100}	Idhn^{145}	Idhn^{100}	Idhn^{227}
G-3-PDH	G-3-pdh^{100}	G-3-pdh^{135}	G-3pdh^{107}	G-3-pdh^{139}
PGI	Pgi^{100}	Pgi^{100}	Pgi^{100}	Pgi^{100}
LAP	Lap^{100}	Lap^{100}	Lap^{100}	Lap^{100}

willistoni group exhibit an average genetic distance of approximately $D = 1.06$ (Avise, 1976) and the average genetic distance among 19 species of *Rhagoletis* fruit flies is $D = 1.08$ (Berlocher and Bush, 1982).

Culliney (1983) argued, based upon the fossil record, for a time of origin and radiation for the genus of 35 mya, with a subsequent decline in species diversity, perhaps brought on by Pleistocene glaciation. This time of origin is in agreement with divergence times estimable from the Nei distances found in this study. He further speculates that *A. mellifera* and *A. cerana* are relatively new species (or a single species?) that may have originated during the Quaternary (approx. 2 mya) as species adapted to colder climates. Ruttner (1988) has recently reviewed considerable evidence concerning the evolution of *Apis* and, based on morphology, behavior and venom protein (mellitin) sequence differences, also concludes that *A. mellifera* and *A. cerana* are recent species, *e.g.* " (Pleistocenic)". However, electrophoretic data from the present study do not support such a recent divergence of *A. cerana* and *A. mellifera*. The amount of genetic change that has accumulated between *A. mellifera* and *A. cerana* ($D = 1.10$) is almost as extensive as that between *A. dorsata* and *A. florea* ($D = 1.40$), indicating that the *mellifera-cerana* split (*cladogenesis*) is probably closer in geological age to the *dorsata–florea* cladogenesis than to the Quaternary. The identical sequence of the mellitin protein (26 amino acids) in *A. cerana* and *A. mellifera*, must be considered in light of data from 18 proteins (enzymes) from the current study. The resolution of the apparent discrepancy between our electrophoretic results and conclusions based on morphology and behavior, may involve the allopatric distribution of *A. cerana* and *A. mellifera*. For example, once allopatry was established (presumably near the time of cladogenesis of the two species), directional selection for further morphometric and behavioral divergence may have been reduced or eliminated. Therefore, the rate of morphological and behavioral evolution in the two species (at least as regards divergence from each other) could have slowed considerably relative to the sympatric extant open-nesting species. This would not be unlike the evo-

lutionary "stagnation" described for extinct European *A. armbrusteri* and extant Indo-Malayan *A. dorsata*, species separated by 12 million years that overlap completely in factor analysis clusters of wing characters (Ruttner, 1988). If further support for the hypothesis of an earlier divergence of *cerana* and *mellifera* is forthcoming, then it becomes more likely that cavity-nesting originated independently in the evolution of the two species (especially if this trait arose during glaciation).

The debate over the probable phylogeny of the genus is not readily resolved by the data in this study. Based on a simple cladistic analysis (Fig. 2), all three possible unrooted trees for the four taxa are supported by the data equally well (or poorly). The UPGMA analysis, on the other hand (Fig. 1), does support an earlier divergence time for *A. florea* than any of the other species, but fails to resolve the *dorsata—cerana—mellifera* group. Because of the large number of enzymes showing fixed differences among the species we may be close to, if not beyond, the resolving power of enzyme electrophoresis. There are, however, several possibilities for further resolution of *Apis* phylogeny. First, an electrophoretic survey of more loci than were assayed in the present study may provide additional useful characters for analysis. That is, if additional phylogenetically-informative loci are found, it is likely that a pattern will emerge indicating phylogenetic relationships. Moreover, extensive sampling of *Apis* species, throughout their ranges, is necessary to measure accurately the amount and kind of enzyme polymorphism present. Sri Lanka is an island country and our samples of *A. cerana*, *A. dorsata* and *A. florea* certainly represent only a small portion of the genetic diversity of the species. Additional electrophoretic studies on these and other *Apis* species from different areas of their endemic range are being conducted (G. Otis, personal communication) and should prove valuable in assessing the potential of enzyme electrophoresis for phylogeny estimation in the genus. Finally, the use of molecular techniques for examining genes in more detail, such as restriction endonuclease mapping or sequencing, may be necessary to resolve the phylogeny.

The results of the present study do indicate that electrophoresis will be a powerful tool for the discrimination of disputed species in *Apis,* since it so readily differentiated the species we examined. Thus, the resolution of the *A. dorsata* "complex", including the corroboration of *A. laboriosa* and others as valid species (as urged by Sakagami *et al.,* 1980; Roubik *et al.,* 1985), seems imminently solvable by electrophoresis. Although an individual colony represents, at best, a tiny "population", data entry of individual colonies as populations in the BIOSYS program still provides unambiguous species discrimination.

The treatment of electrophoretic data with phylogenetic tree-building or clustering techniques also appears to have promise for the discrimination of endemic subspecies or races in *Apis mellifera* (and probably other *Apis*), as can be seen in Fig. 3. The five colonies of *A. m. mellifera* (Norway) are clearly discriminated from the five colonies of Italian (*A. m. ligustica*) and five Czechoslovakian (predominately *A. m. carnica*) colonies. However, based on the electrophoretic data, the Italian and Czechoslovakian samples are indistinguishable. These races are generally quite similar in appearance (Ruttner, 1975) and may be genetically similar, but additional samples from these races need to be investigated. This is particularly true since the *A. m. carnica* used in this study may not represent pure *carnica* (Sheppard and McPheron, 1986). Whether electrophoresis of enzymes will be useful in discriminating the

racial "ancestry" of current populations of *Apis mellifera* in the New World will depend upon the presence of sufficient measurable genetic variation in New World honey bees. Unfortunately, the honey bees from the United States appear to be depauperate in enzyme polymorphism compared to even the few European colonies that have been surveyed (Sylvester, 1976; Nunamaker, 1980; Sheppard, 1988). Therefore, the development of a reliable method of ancestral or hybrid racial identification within species will likely depend on the application of molecular techniques that are more sensitive to genomic variation than is enzyme electrophoresis.

Résumé — Différenciation des 4 espèces d'*Apis* d'après la variation allozymique. Les allozymes sont des enzymes dont 2 ou plusieurs types sont liés génétiquement; ils sont mis en évidence par l'électrophorèse. Le but de ce travail est de comparer du point de vue électrophorétique les 4 espèces d'*Apis* les plus connues et d'évaluer, à l'aide des données obtenues, leurs relations phylogénétiques et le degré de différenciation. A l'aide de la technique d'électrophorèse séquentielle on a réalisé un inventaire des 18 loci d'enzymes d'*Apis cerana, A. dorsata* et *A. florea* du Sri Lanka. Il s'agit des enzymes suivantes (entre parenthèses nos abréviations et les numéros de la Commission des Enzymes) : aconitase (ACON; 4.2.1.2), aldolase (ALDO; 4.1.2.13), alpha-glycérophosphate déshydrogénase (α-GDH; 1.1.1.8), arginine kinase (ARGK; 3.3.8.9), acide bêta-hydroxybutyrique déshydrogénase (β-HDBH; 1.1.1.30), estérase (EST; 3.1.1.1), glycéraldéhyde-3-phosphate déshydrogénase (G-3-PDH; 1.2.1.12), hexokinase (HEX. 2.7.1.1), isocitrate déshydrogénase (IDH; 1.1.1.42), leucine aminopeptidase (LAP; 3.4.1.1), malate déshydrogénase (MDH; 1.1.1.37), enzyme malique (ME; 1.1.1.40), phosphoglucomutase (PGM; 2.7.5.1), phosphoglucose isomérase (PGI; 5.3.1.9) et triose phosphate isomérase (TPI; 5.3.1.1). Les données publiées concernant l'abeille européenne *A. mellifica* sont incluses dans cette étude. Sept loci d'enzymes présentent un polymorphisme et, pour certaines espèces, c'est la première fois qu'ils sont mentionnés. Sept des loci présentent une différence allozymique fixée chez les 4 espèces et 3 loci sont fixés chez les 4 espèces pour le même allozyme. Les données concernant les allozymes ont été analysées par la méthode de regroupement UPGMA, par le procédé de la distance de Wagner et par une méthode cladistique simple. La distance génétique moyenne (Nei's D = 1,30) montre que des différences allozymiques considérables se sont accumulées parmi les espèces. En raison de la distance génétique relativement grande qui existe entre *A. cerana* et *A. mellifica* (D = 1,10), les données électrophorétiques ne corroborent pas, pour ces espèces, une date de divergence aussi récente que celle suggérée par les caractères morphologiques et éthologiques. Les données électrophorétiques ne résolvent pas les questions concernant la phylogenèse d'*Apis*, en raison surtout du nombre de différences fixées parmi les espèces. L'électrophorèse se montre prometteuse pour la détermination des espèces, la résolution du "complexe" *dorsata* et quelques autres problèmes concernant *Apis,* puisque même l'entrée de données d'une seule colonie dans le programme Biosys a fourni une discrimination univoque. Le traitement des données électrophorétiques par les techniques d'arborisation ou de regroupement phylogénétiques présente des possibilités pour déterminer les formes endémiques d'*A. mellifica.*

Zusammenfassung — Unterscheidung der vier *Apis*-Arten auf Grund der Variation von Allozymen. Allozyme sind Enzyme, von denen durch Elektrophorese 2 oder mehr genetisch bedingte Typen gefunden wurden. Es ist das Ziel dieser Untersuchung, die 4 am besten bekannten *Apis*-Arten elektrophoretisch miteinander zu vergleichen und an Hand der gewonnenen Daten phylogenetische Verwandtschaften und den Grad der Differenzierung zwischen den Arten abzuschätzen. Mit Hilfe der Technik der sequentiellen Elektrophorese wurde eine Bestandsaufnahme von 18 Enzym-Loci von *Apis cerana, A. dorsata* und *A. florea* aus Sri Lanka durchgeführt. Es handelte sich um folgende Enzyme (unsere Abkürzungen und die Nummer der Enzym-Kommission in Klammer) : Aconitase (ACON; 4.2.1.2), Aldolase (ALDO; 4.1.2.13), Alpha-glycerophosphat Dehydrogenase (α-GDH; 1.1.1.8), Arginin kinase (ARGK; 3.3.8.9), Beta-hydrocybutyric acid Dehydrogenase (β-HBDH, 1.1.1.30), Esterase (EST; 3.1.1.1), Glyceraldehyd-3-Phosphat-Dehydrogenase (G-3-PDH; 1.2.1.12), Hexokinase (HEX; 2.7.1.1), Isocitrat Dehydrogenase (IDH; 1.1.1.42), Leucin Aminopeptidase (LAP; 3.4.1.1), Malat Dehydrogenase (MDH; 1.1.1.37), Malic Enzym (ME; 1.1.1.40), Phosphoglucomutase (PGM; 2.7.5.1), Phosphoglucose Isomerase (PGI; 5.3.1.9) und Triose Phosphat Isomerase (TPI; 5.3.1.1.). In dieser Untersuchung wurden publizierte Daten von europäischer *A. mellifera* einbezogen. Sieben Enzym-Loci zeigten Polymorphismen, über die für einige hier zum ersten Mal berichtet wird. Sieben der Loci wiesen fixierte Allozym-Unterschiede bei allen 4 Arten auf und 3 Loci waren bei allen 4 Arten für dasselbe Allozym fixiert. Die Allozym-Daten wurden nach der UPGMA-Clustermethode, dem Distanz-Wagner-Verfahren und einer einfachen cladistischen Methode analysiert. Die mittlere genetische Distanz (Nei's D = 1,30) weist darauf hin, daß sich zwischen den Arten erhebliche Allozym-Unterschiede angesammelt haben. Wegen der relativ großen genetischen Distanz zwischen *A. cerana* und *A. mellifera* (D = 1.10) kann gesagt werden, daß die elektrophoretischen Daten keinen so rezenten Zeitpunkt der Divergenz stützen, wie dies auf Grund morphologischer und Verhaltens-Merkmale angenommen wird. Die elektrophoretischen Daten leisten keinen Beitrag zur Lösung von Fragen der Phylogenie, vor allem deshalb, weil eine so große Zahl der Unterschiede zwischen den Arten fixiert ist. Elektrophorese erscheint vielversprechend für die Bestimmung von Arten, die Auflösung des *A. dorsata*-"Komplex" und für andere Probleme bei Apis, da sogar die Eintragung von Daten eines einzigen Volkes in das BIOSYS-Programm zu einer eindeutigen Unterscheidung geführt hat. Die Bearbeitung von elektrophoretischen Daten mit Techniken, die zu phylogenetischen Stammbäumen und Clustern führen, birgt Möglichkeiten zur Bestimmung von endemischen Formen von *A. mellifera*.

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