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A NEW SPECIES WITHIN THE *ANOPHELES PUNCTULATUS* COMPLEX IN WESTERN PROVINCE, PAPUA NEW GUINEA

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ABSTRACT. Specimens identified morphologically as *Anopheles punctulatus* revealed 2 genetically distinct entities in samples collected in the Western Province of Papua New Guinea. We regard one of these species as conspecific with *An. punctulatus* and the other as a new species designated here as *An.* sp. near *punctulatus.*

INTRODUCTION

The members of the *Anopheles punctulatus* Dönitz complex are important vectors of malaria and bancroftian filariasis in the southwest Pacific (Lee et al. 1987). The number of species revealed within this complex has increased over the past 2 decades. In 1973, 4 species were delimited by crossmating (Bryan 1973): *An. punctulatus* in which the apical half of the proboscis of the female is usually pale, *Anopheles koliensis* Owen with more restricted pale marking on the proboscis of some specimens, and 2 species with a dark proboscis (except for a subapical pale ring), a feature once considered diagnostic for *Anopheles farauti* Laveran *sensu lato* (*s.l.*). These cies have been given the non-Linnean designations *An. farauti* No. 1 and *An. farauti* No. 2 (Bryan 1973). *Anopheles farauti* No. 1 is widespread, being recorded from Papua New Guinea (Foley et al. 1993), northern Australia (Mahon et al. 1981; Sweeney et al. 1990; Foley et al. 1991a, 1991b), the Solomon Islands and Vanuatu (Bryan 1973, 1981; Mahon 1983; Hii et al. 1993; Foley et al. 1994) and is *An. farauti sensu stricto* (*s.s*) (Hii et al. 1993, Foley et al. 1994). *Anopheles farauti* No. 2 has been recorded in northern Australia (Bryan 1973, Mahon et al. 1981, Sweeney et al. 1990, Foley et al. 1991b) and the Solomon Islands (Foley et al. 1994). Chromosomal and cross-mating studies also revealed the presence of the species designated *An. farauti* No. 3 (Mahon et al. 1981, Mahon and Miethke 1982), which has only been reported from Australia (Mahon and Miethke 1982; Sweeney et al. 1990; Foley et al. 1991a, 1991b). Three additional species, *An. farauti* No. 4, No. 5, and No. 6, have been detected in Papua New Guinea (Foley et al. 1993) and one, *An. farauti* No. 7, in the Solomon Islands (Foley et al. 1994) through allozyme analysis. A further 2 species have been recognized solely on morphology. *Anopheles clowi* was described by Rozeboom and Knight (1946) from specimens collected near Jayapura (Irian Jaya, Indonesia) and it is only known from that area. Specimens from the Rennell Islands that are morphologically intermediate between *An. farauti s.l.* and *Anopheles koliensis* (Maffi 1973), have been formally named *Anopheles rennellensis* (Taylor and Maffi 1991). The relationship between the morphologically defined species (*An. clowi* and *An. rennellensis*) and those defined by allozyme, chromosomal, and cross-mating studies is unknown.

Species within the *An. punctulatus* complex cannot be reliably separated on their external morphology. *Anopheles farauti ss.,* No. 2, and No. 3 can be distinguished by the banding pattern of their larval salivary gland polytene chromosomes (Mahon 1983), by allozymes (Mahon 1984), and by DNA probes (Booth et al. 1991, Cooper et al. 1991, Hartas et al. 1992). DNA probes have also been developed for *An. farauti* No. 4, No. 5, and No. 6, and *An. koliensis* and *An. punctulatus* (Beebe et al. 1994). However, because of the possibility that additional, as yet undetected, species exist within this group of mosquitoes, we undertake allozyme analysis in previously unstudied areas to determine species composition. In this paper we report the results of an allozyme electrophoretic study of specimens collected in the Western Province of Papua New Guinea.

MATERIALS AND METHODS

In April and May 1992 anopheline mosquitoes were collected from a number of locations throughout the Western Province of Papua New Guinea (Fig. 1). Specimens were collected as adults using CO_2-baited light traps or as larvae that were reared to the adult stage. Where possible, specimens were identified morphologically using the keys of Lee et al. (1987). Specimens from 5 localities (Table 1) belonging to the *An. punctulatus* complex were then subjected to allozyme analysis. Reference specimens of *An. farauti s.s.,* No. 2, and No. 3, which were obtained from colonies whose provenance is detailed in Foley et al. (1993), were included on each gel.

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Electrophoresis and allozyme analysis: Electrophoresis was performed using cellulose acetate plates (Helene Laboratories, Beaumont, TX, USA) and buffers, stains, and running conditions as described in Foley et al. (1993, 1994). Allozyme data were collected from individual specimens with a maximum of 33 loci from one specimen being studied. These loci were: ACON-1, ACON-2, ACP, AK-2, FDP-2, βGAL-1, βGAL-2, GOT-1, GOT-2, αGPD, 6GPD, GPI, HBDH, HK-1, HK-2, HK-3, IDH-1, IDH-2, LDH, MDH-1, MDH-2, ME-1, ME-2, MPI-2, ODH, PEPB-1, PEPB-2, PEPD-1, PEPD-2, PGK, PGM, PK, and THDH. For analysis, data from individuals were pooled according to location, with pools regarded as single operational taxonomic units (OTUs) except if noninterbreeding sympatric species were identified by the existence of fixed genetic differences. Data were analyzed by the unweighted pair group method of Sneath and Sokal (1973) to obtain the percentage of loci for which OTUs do not share alleles and Nei's D, corrected for small sample size (Nei 1978). Data from known *An. farauti s.s.*, No. 2, No. 3, No. 4, No. 5, No. 6, and No. 7, and *An. koliensis* and *An. punctulatus* were also included in the analysis (Table 1).

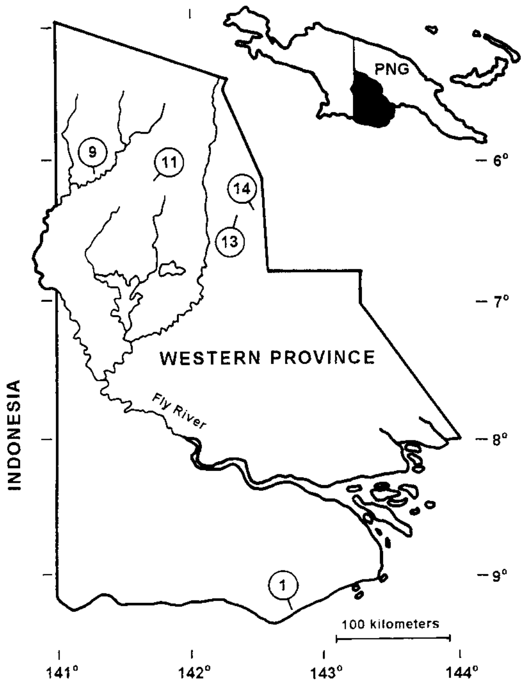

Fig. 1 Collection sites for operational taxonomic units within the *Anopheles punctulatus* complex in the Western Province of Papua New Guinea. See Table 1 for site and collection details.

RESULTS

A total of 156 specimens in 14 OTUs were analyzed (Table 1). Nei's D and percent fixed differences between pairs of OTUs are given as

Table 1. The operational taxonomic unit (OTU) number, sample size (in parentheses), collection locality, and identification for members of the *Anopheles punctulatus* complex in the Western Province of Papua New Guinea (PNG), plus five OTUs from 3 sites in PNG and one from the Solomon Islands (SI). OTUs marked * were collected as larvae, those marked # were collected landing on humans.

OTU	Locality	Coordinates	Identification
		Western Province	
1 (8)	Daru District (coastal)*	142°46′E, 9°15′S	*An. farauti s.s.*
9 (2)	Kiunga Township*	141°18′E, 6°07′S	*An. koliensis*
11 (6)	IJ Refugee Camp*	141°27′E, 6°11′S	*An. punctulatus*
13 (9)	Rentoul River*	142°30′E, 6°24′S	*An.* sp. near *punctulatus*
14 (5)	Rentoul River*	142°38′E, 6°22′S	*An.* sp. near *punctulatus*
		Other	
2 (10)	Colony Standard		*An. farauti s.s.*
3 (10)	Colony Standard		*An. farauti* No. 2
4 (10)	Colony Standard		*An. farauti* No. 3
5 (8)	Hudini# (PNG)	145°45′E, 5°17′S	*An. farauti* No. 4
10 (13)	Hudini# (PNG)	145°45′E, 5°17′S	*An. koliensis*
12 (26)	Hudini# (PNG)	145°45′E, 5°17′S	*An. punctulatus*
6 (20)	Goroka# (PNG)	145°20′E, 6°01′S	*An. farauti* No. 5
7 (14)	Tari# (PNG)	145°52′E, 5°57′S	*An. farauti* No. 6
8 (15)	Mamara* (SI)	159°53′E, 9°24′S	*An. farauti* No. 7

Table 2. Genetic difference matrix for operational taxonomic units (OTUs) of the *Anopheles punctulatus* complex in the Western Province of Papua New Guinea, showing percentage fixed differences below the diagonal and genetic distance (Nei 1978) above the diagonal. The OTUs connected by a horizontal bar are grouped 1–10 according to genetic similarity (see text).

Group	1		2	3	4	5	6	7	8		9		10	
OTU	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0	0.08	0.44	0.43	0.98	0.63	0.51	0.27	0.57	0.51	0.68	0.60	0.67	0.72
2	6	0	0.39	0.34	0.91	0.52	0.46	0.21	0.62	0.59	0.63	0.55	0.74	0.83
3	30	33	0	0.45	0.99	0.42	0.38	0.28	0.48	0.60	0.69	0.58	0.69	0.76
4	27	30	36	0	1.20	0.57	0.44	0.40	0.54	0.52	0.74	0.73	0.89	0.92
5	53	53	63	63	0	0.94	1.02	0.87	0.80	0.86	0.92	0.89	1.01	1.08
6	39	39	33	39	50	0	0.44	0.42	0.69	0.72	0.81	0.73	0.55	0.60
7	33	33	27	27	50	27	0	0.44	0.65	0.63	0.73	0.65	0.83	0.91
8	18	18	21	30	50	30	30	0	0.49	0.52	0.76	0.61	0.73	0.80
9	37	47	37	43	48	50	37	37	0	0.06	0.98	0.91	0.54	0.51
10	27	39	33	33	53	42	30	36	0	0	0.94	0.93	0.53	0.54
11	47	47	50	50	55	53	43	50	55	53	0	0.03	0.50	0.52
12	36	33	36	45	56	42	36	33	50	48	0	0	0.57	0.61
13	44	47	44	47	55	31	41	41	33	31	34	38	0	0.00
14	52	58	52	58	60	39	52	48	33	32	38	39	0	0

a matrix in Table 2 and percent fixed differences are illustrated in Fig. 2. The dendrogram for Nei's D is not shown as it was congruent with percent fixed differences. Allele frequencies are shown in Table 3 except for ACON-2, AK-2, GOT-1, αGPD, IDH-2, and PGK, which were monomorphic; alleles were identical to the colony *An. farauti s.s.* pattern in all specimens (see Foley et al. 1993). The OTUs from the Western Province fall into 4 groups with greater genetic similarity within groups than between groups. Percent fixed differences and Nei's D values within groups were 0–6% and 0.00–0.08, respectively, whereas between-group values were 18–63% and 0.21–1.20, respectively (Table 2). The OTUs within groups are regarded as conspecific. Allozyme patterns of 3 of these groups conform to those of the previously recognized species *An. farauti s.s.*, *An. koliensis*, and *An. punctulatus* (Foley et al. 1993). The remaining group was composed of 2 OTUs (13, 14) in which specimens were morphologically similar to *An. punctulatus* with extensive pale scaling on the apical half of the proboscis. However, they were genetically very distinct from

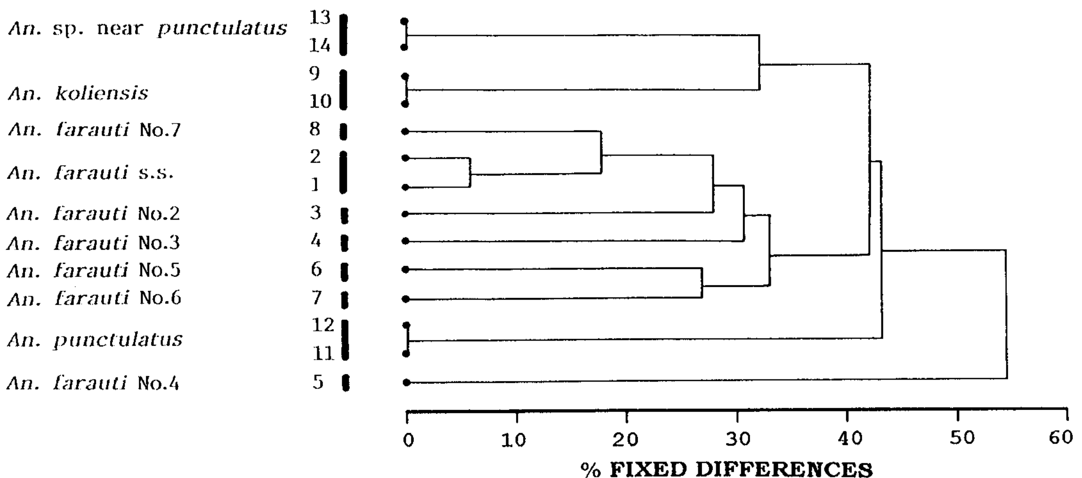

Fig. 2. Phenogram of percentage fixed differences constructed by the unweighted pair group method for operational taxonomic units of the *Anopheles punctulatus* complex in the Western Province of Papua New Guinea.

Table 3. Allele frequencies for *Anopheles farauti s.s.* (AF), *Anopheles koliensis* (AK), *Anopheles punctulatus* (AP), and *Anopheles* sp. near *punctulatus* (P2) in the Western Province of Papua New Guinea. Allelic designations follow Foley et al. (1993, 1994). *n* = number of individuals, <a = band slower than a. Electromorphs of most value for discriminating P2 (relative to AF1, AK, and AP) are underlined.

Locus	AF	AK	AP	P2
ACON-1				
n	8	2	2	14
a	1.00	1.00	0.00	0.00
b	0.00	0.00	1.00	1.00
ACP				
n	7	2	6	13
a	0.64	0.00	0.00	0.00
c	0.00	0.50	1.00	1.00
d	0.21	0.50	0.00	0.00
e	0.15	0.00	0.00	0.00
FDP-2				
n	5	2	3	11
a	1.00	1.00	0.00	1.00
b	0.00	0.00	1.00	0.00
BGAL-1				
n	8	2	6	14
<a	0.00	0.00	1.00	1.00
a	0.75	0.00	0.00	0.00
b	0.25	0.00	0.00	0.00
c	0.00	1.00	0.00	0.00
BGAL-2				
n	7	2	6	14
b	1.00	0.00	0.00	0.07
c	0.00	1.00	0.00	0.93
d	0.00	0.00	1.00	0.00
GOT-2				
n	4	2	4	10
b	0.00	1.00	1.00	1.00
c	0.50	0.00	0.00	0.00
d	0.50	0.00	0.00	0.00
6PGD				
n	8	2	6	14
a	0.00	0.50	0.00	0.00
b	1.00	0.50	1.00	1.00
GPI				
n	6	2	6	13
a	1.00	1.00	0.00	0.00
b	0.00	0.00	1.00	0.92
c	0.00	0.00	0.00	0.08
HBDH				
n	5	1	6	8
b	1.00	1.00	0.00	1.00
c	0.00	0.00	1.00	0.00
HK-1				
n	8	2	2	14
a	0.00	0.00	0.00	1.00
b	1.00	0.00	1.00	0.00
d	0.00	1.00	0.00	0.00
HK-2				
n	8	2	2	14
a	0.00	0.00	0.00	1.00
b	1.00	0.00	1.00	0.00
c	0.00	1.00	0.00	0.00
HK-3				
n	8	2	2	14
b	0.00	0.00	0.00	1.00
c	1.00	0.00	1.00	0.00
e	0.00	1.00	0.00	0.00
IDH-1				
n	4	2	2	13
b	0.88	1.00	1.00	1.00
c	0.12	0.00	0.00	0.00
LDH				
n	5	2	2	11
a	1.00	0.00	0.00	0.00
c	0.00	1.00	0.00	0.91
d	0.00	0.00	1.00	0.09
MDH-1				
n	6	2	6	13
b	1.00	1.00	0.00	0.00
c	0.00	0.00	1.00	1.00
MDH-2				
n	2	0	0	4
b	1.00	0.00	0.00	0.75
c	0.00	0.00	0.00	0.25
ME-1				
n	7	2	6	14
a	0.00	0.00	1.00	0.00
d	1.00	1.00	0.00	1.00
ME-2				
n	4	2	2	7
a	1.00	0.00	0.00	0.00
b	0.00	0.00	0.00	1.00
c	0.00	0.00	1.00	0.00
d	0.00	1.00	0.00	0.00

Table 3. Continued.

Locus	AF	AK	AP	P2
MPI-2				
n	8	2	6	14
c	0.13	0.00	0.00	0.07
<d	0.43	0.25	0.25	0.00
d	0.38	0.00	0.00	0.14
e	0.00	0.50	0.58	0.79
f	0.06	0.25	0.17	0.00
ODH				
n	7	0	0	12
b	1.00	0.00	0.00	0.00
d	0.00	0.00	0.00	1.00
PEPB-1				
n	2	2	0	6
c	0.00	1.00	0.00	0.00
d	1.00	0.00	0.00	1.00
PEPB-2				
n	8	2	6	14
c	1.00	0.75	0.92	1.00
d	0.00	0.25	0.00	0.00
PEPD-1				
n	4	2	4	11
<a	0.00	0.00	0.00	1.00
a	0.13	0.00	0.00	0.00
b	0.25	1.00	0.00	0.00
c	0.62	0.00	1.00	0.00
PEPD-2				
n	4	2	4	6
b	1.00	1.00	0.38	0.83
c	0.00	0.00	0.12	0.17
d	0.00	0.00	0.50	0.00
PGM				
n	4	2	6	11
b	0.38	1.00	0.00	0.86
c	0.62	0.00	0.00	0.14
d	0.00	0.00	1.00	0.00
PK				
n	5	2	6	13
a	1.00	0.00	1.00	0.89
c	0.00	1.00	0.00	0.11
THDH				
n	8	2	6	14
c	0.00	0.50	0.00	0.00
d	0.00	0.50	1.00	1.00
e	1.00	0.00	0.00	0.00

An. punctulatus collected in the Western and Madang Provinces (OTUs 11 and 12, respectively), with fixed genetic differences of 34–39% and Nei's D ranging from 0.50 to 0.61. The maximum genetic divergence recorded within *An. punctulatus* collected from widely separated localities in Papua New Guinea was 18% (Foley et al. 1993), and 6% for specimens collected in the Solomon Islands (Foley et al. 1994). Operational taxonomic units 13 and 14 also differed greatly from *An. koliensis*, *An. farauti s.s.*, and *An. farauti* No. 2, No. 3, No. 4, No. 5, No. 6, and No. 7 (Table 2 and Fig. 2), indicating that they are not conspecific with any of these species. Furthermore, specimens in OTUs 13 and 14 do not compare to the morphological description of *An. clowi* or *An. rennellensis*, strongly supporting the hypothesis that these specimens belong to a previously unrecognized species. We propose that until a taxonomic review of the *An. punctulatus* group is made, this species should be designated as *Anopheles* species near *punctulatus*. Specimens of this species were homozygous for a unique allele at Pep D-1 (Table 3). Although numbers were low, this locus may be useful to separate this species from the rest of the complex; the allele in *An.* sp. near *punctulatus* is slower than those of the other species. Additional alleles that will discriminate between *An. punctulatus* and *An.* sp. near *punctulatus* are at HK, ME-1, and HBDH.

DISCUSSION

The presence of a species that is morphologically very similar to *An. punctulatus* requires a reassessment of data assigned to *An. punctulatus s.s.* and sounds a cautionary note for those working with this group of mosquitoes. Electrophoretic keys given in Foley and Bryan (1993) require modification to accommodate the newly discovered species. For example, in Key 1 and Key 2, couplet 1 will separate *An.* sp. near *punctulatus* and *An. farauti* No. 5 from other members of the complex. These 2 species can be separated by proboscis coloration (all dark in *An. farauti* No. 5, apical half pale in *An.* sp. near *punctulatus*) or relative migration of HBDH bands (*An.* sp. near *punctulatus* has the standard pattern whereas *An. farauti* No. 5 is faster). Mis-

identifications could result from using Keys 3 and 4 and their use is not recommended where specimens fitting the description of *An. punctulatus* occur, until the distribution of *An.* sp. near *punctulatus* has been established. As stated in Foley and Bryan (1993), greater sampling of field populations may reveal overlap between previously "diagnostic" enzymes, which should therefore be regarded as provisional. The discovery of yet another species within this group demonstrates the importance of testing for cryptic species in new study areas.

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