CHAPTER 3

Results

3.1 Molecular species identification

Species identification, based on molecular analyses (cytochrome *c* oxidase subunit I barcoding region, 658 bp), was generated from a total of 40 specimens of the F_1-progenies of the 9 species, i.e., using one specimen from each of five iso-female lines of each species. The NJ and Bayesian trees revealed eight distinct clusters. Each species (n=5) showed a low level of mean intra-specific genetic distance (0.001-0.009), whereas, the mean inter-specific genetic distance of each was high (0.032-0.077) (Table 3.1). Among the species examined, the NJ tree revealed two major clades (Figure 3.1). Clade I comprised *An. crawfordi*, *An. paraliae*, *An. peditaeniatus*, *An. sinensis* and a member of the Korean Hyrcanus Group, *An. lesteri*. In this clade, the five species were separated from each other, with high bootstrap support (98-100%). The topology of the clade reveals that *An. paraliae* is more closely related to *An. peditaeniatus* (mean genetic distance = 0.038) than to *An. crawfordi* (mean genetic distance=0.041). In addition, *An. paraliae* shows closer proximity to *An. sinensis* (mean genetic distance = 0.032) than to *An. crawfordi* (mean genetic distance = 0.047). Remarkably, a low level of mean genetic distance (0.011) between *An. paraliae* and *An. lesteri* was observed in this study. Clade II, comprised of *An. argyropus*, *An. nigerrimus*, *An. nitidus* and *An. pursati*, is supported by a high bootstrap value of 90%. Within this clade, *An. nitidus* is more closely related to *An. pursati* (mean genetic distance = 0.039) than to *An. nigerrimus* (mean genetic distance = 0.049). Additionally, *An. nitidus* is more closely related to *An. argyropus* (mean genetic distance = 0.054) than to the other species. *Anopheles crawfordi* and *An. pursati* are most genetically diverse, with mean genetic distances of 0.077. The topology of the Bayesian tree (Figure 3.2) is similar to the topology to the NJ tree, but consists of four clades that form an unresolved polytomy.Four species, *An. argyropus*, *An. nigerrimus*, *An. nitidus* and *An. pursati*,

comprise a clade with a high posterior probability (99%). *Anopheles paraliae* and *An. lesteri* form a clade that has moderate support (posterior probability = 70%). *Anopheles peditaeniatus* comprises the third clade and the fourth consists of *An. crawfordi* and *An. sinensis* as weakly supported sister species. The support for the conspecificity of the specimens of each species is strongly supported, e.g. *An. pediataeniatus* = 97.

Table 3.1 Mean intra- and inter- specific genetic distances (K2P) for eight species of the Hyrcanus Group based on COI barcode sequences

Species	Mean intra-specific genetic distances (n = 5)	1	2	3	4	5	6	7
1. *An. argyropus*	0.004							
2. *An. crawfordi*	0.002	0.069						
3. *An. nigerrimus*	0.002	0.057	0.061					
4. *An. nitidus*	0.009	0.054	0.075	0.049				
5. *An. paraliae*	0.001	0.059	0.041	0.050	0.058			
6. *An. peditaeniatus*	0.005	0.069	0.057	0.067	0.059	0.038		
7. *An. pursati*	0.006	0.058	0.077	0.050	0.039	0.062	0.063	
8. *An. sinensis*	0.002	0.061	0.047	0.062	0.059	0.032	0.048	0.076

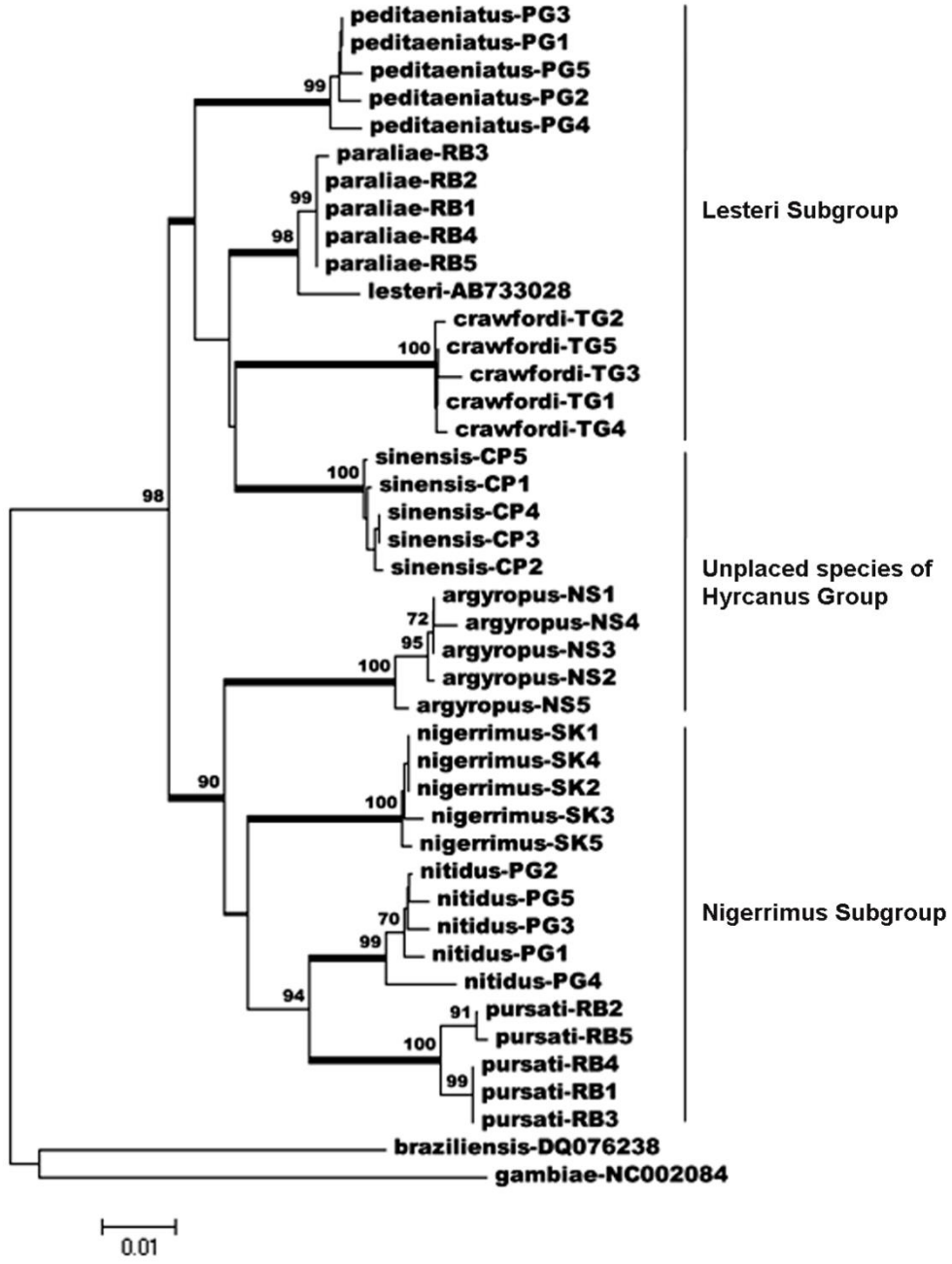

Figure 3.1 Bootstrapped neighbor-joining tree (NJ) of the COI barcode sequences of nine species of the Hyrcanus Group. *Anopheles gambiae* and *Anopheles braziliensis* were used as outgroup taxa. Bootstrap values higher than 70% are shown above the node. Bars represent 0.01 substitutions per site

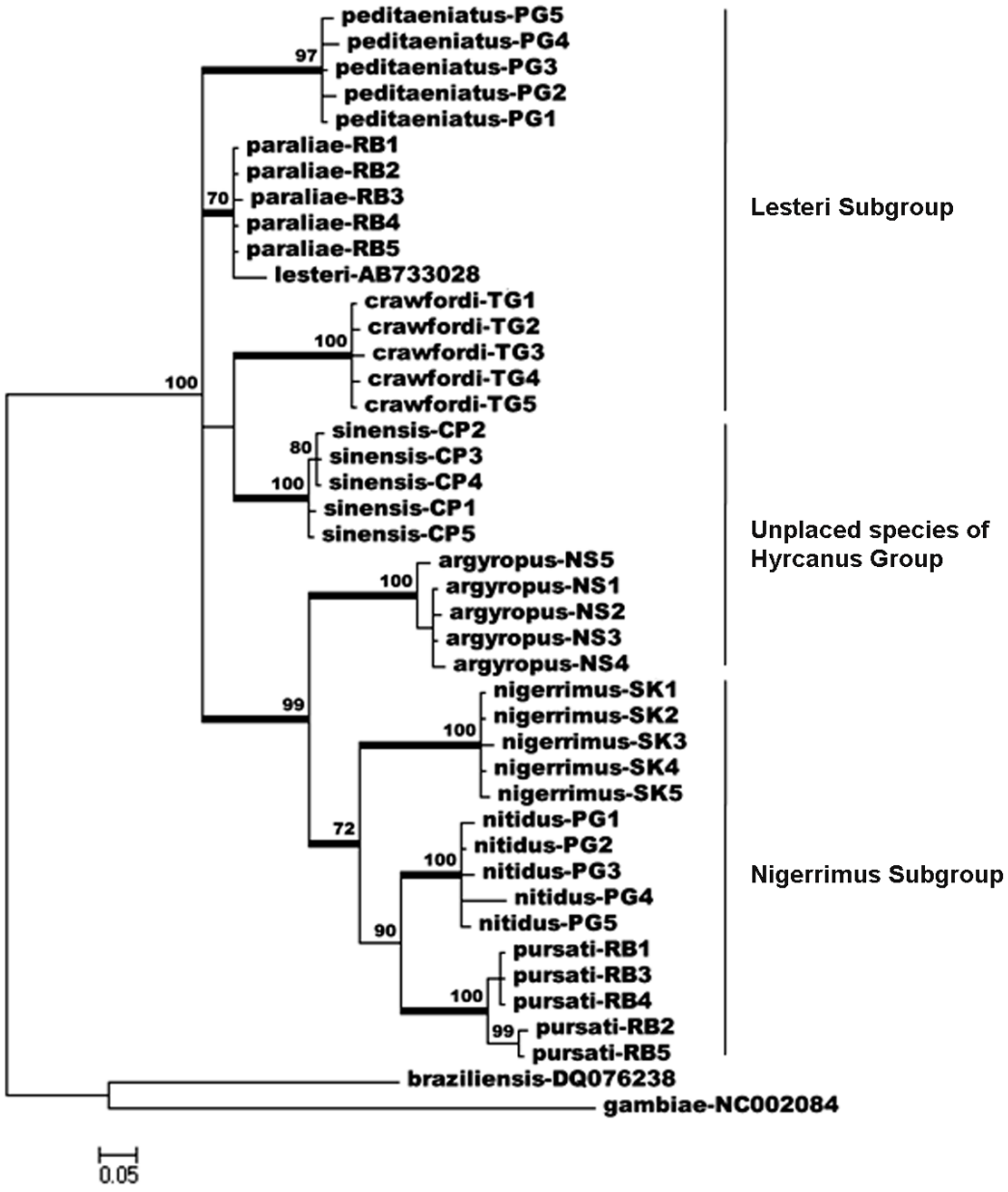

Figure 3.2 Bayesian phylogenetic tree based on COI barcode sequences of nine species of the Hyrcanus Group. *Anopheles gambiae* and *An. braziliensis* were used as outgroup taxa. The posterior probabilities higher than 70% is shown above the nodes. Bars represent 0.05 substitutions per site

3.2 Establishment of a stock colony

Eight species of the Hyrcanus Group, i.e., *An. argyropus* (F_{23}), *An. crawfordi* (F_{19}), *An. nigerrimus* (F_{15}), *An. nitidus* (F_{16}), *An. paraliae* (F_{16}), *An. peditaeniatus* (F_{29}), *An. pursati* (F_{12}) and *An. sinensis* (F_{11}), were reared successfully for many consecutive generations by using artificial mating techniques. The developmental time of one generation (from eggs to eggs) was more or less than 28 days, comprising a duration of 3-4 (3), 8-14 (11) and 1-3 (2) days of egg, larval and pupal development, respectively, as well as 5 days for blood feeding and 7 days for maturation and ovipositioning by females.

3.3 Screening of stenogamous behavior and establishment of self-mating colonies

The results of natural mating by 200 and 300 newly emerged female and male F_9 mosquitoes, respectively, in a 30 x 30 x 30 cm cage for 1 week indicated that females of only five species, i.e., *An. pursati*, *An. sinensis*, *An. nigerrimus*, *An. paraliae* and *An. peditaeniatus*, could oviposit successfully, with insemination rates of 31%, 33%, 42%, 50% and 77%, respectively (Table 3.2). The remaining three species, *An. argyropus*, *An. crawfordi* and *An. nitidus*, failed to copulate in the laboratory cages. After selection, the stenogamous colony of *An. peditaeniatus* was maintained as a self-mating colony for more than 20 generations, with insemination rates that ranged from 61-86%. In contrast, *An. sinensis*, *An. pursati*, *An. nigerrimus* and *An. paraliae* thereafter failed to copulate naturally selection under the same conditions. Therefore, only a self-mating colony of *An. peditaeniatus* was established successfully in this present study.

Table 3.2 The insemination rates of selective stenogamous-colonies of *An. peditaeniatus*, *An. paraliae*, *An. nigerrimus*, *An. pursati* and *An. sinensis*

Mosquito species	**Insemination rates of each selected-generation of passage (F_9-F_{29})***																				
	F_9	**F_{10}**	**F_{11}**	**F_{12}**	**F_{13}**	**F_{14}**	**F_{15}**	**F_{16}**	**F_{17}**	**F_{18}**	**F_{19}**	**F_{20}**	**F_{21}**	**F_{22}**	**F_{23}**	**F_{24}**	**F_{25}**	**F_{26}**	**F_{27}**	**F_{28}**	**F_{29}**
An. peditaeniatus	77	81	75	69	62	83	86	76	61	72	68	64	67	85	66	71	80	72	76	74	79
An. paraliae	50	49	43	45	57	52	56														
An. nigerrimus	42	44	51	46	54	58															
An. pursati	31	27	30																		
An. sinensis	33	34																			

*Examined from 100 spermathecae

3.4 Searching for possible mechanism(s) that control stenogamous mating

3.4.1 Mating ability of adult mosquitoes in various sized cubic cages and a density resting surface (DRS)

1) Mating ability of adult mosquitoes in 10, 20 and 30 cubic cm cages at DRS of 3.6

The comparative mean insemination rates between stenogamous and eurygamous species demonstrated a statistically significant difference ($p < 0.05$) for all cage sizes (Table 3.3). However, *An. peditaeniatus*, which had a high insemination rate, showed no statistically significant difference among cage sizes ($p > 0.05$).

Sperm density in the spermathecae of inseminated female mosquitoes of the eight species was based on grading, using various cage sizes at a DRS of 3.6, which are shown in Table 3.4-3.6. More than 50% of inseminated-female *An. peditaeniatus* showed high sperm density (3+ and 4+) in spermathecae in all cage sizes. Low sperm density (1+) was observed mostly in spermathecae of *An. argyropus*, *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae* and *An. pursati*. The comparative frequency of sperm density, which was high at 3+ and 4+ between *An. peditaeniatus* and the four species, demonstrated a statistically significant difference ($p < 0.05$).

Table 3.3 Insemination rates of the eight species of the Hyrcanus Group using various cage sizes (10, 20 and 30 cubic cm cages) at DRS of 3.6

Mosquito species	Mean insemination rate*		
	10 x 10 x 10 cm**	**20 x 20 x 20 cm****	**30 x 30 x 30 cm****
An. peditaeniatus	88.0	92.0	95.5
An. argyropus	44.5[a]	49.5[a]	65.5[a]
An. crawfordi	0[b]	0[b]	4.0[b]
An. nigerrimus	44.5[c]	67.0[c]	68.0[c]
An. nitidus	9.0[d]	4.0[d]	4.5[d]
An. paraliae	28.0[e]	49.5[e]	41.5[e]
An. pursati	82.0[f]	80.0[f]	87.5[f]
An. sinensis	17.0[g]	23.0[g]	30.0[g]

*Dissected 200 spermathecae/species/cage

**Chi-square test: a, b, c, d, e, f, g vs. *An. peditaeniatus* ($p < 0.05$)

Table 3.4 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (10 cubic cm cage, DRS = 3.6)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	**2+**	**3+**	**4+**
An. peditaeniatus	28	30	52	66
An. argyropus	89	0	0	0
An. crawfordi	0	0	0	0
An. nigerrimus	61	24	2^{a}	2^{a}
An. nitidus	18	0	0	0
An. paraliae	28	4	10^{b}	14^{b}
An. pursati	114	26	12^{c}	12^{c}
An. sinensis	12	2	18^{d}	2^{d}

* Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

Table 3.5 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (20 cubic cm cage, DRS = 3.6)

Mosquito Species*	**Grading of sperm density in female spermathecae****			
	1+	**2+**	**3+**	**4+**
An. peditaeniatus	29	23	35	97
An. argyropus	97	2	0	0
An. crawfordi	0	0	0	0
An. nigerrimus	38	56	11[a]	29[a]
An. nitidus	8	0	0	0
An. paraliae	77	10	9[b]	3[b]
An. pursati	93	26	21[c]	20[c]
An. sinensis	10	0	4[d]	32[d]

* Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

Table 3.6 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (30 cubic cm cage, DRS = 3.6)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	2+	3+	4+
An. peditaeniatus	9	37	28	21
An. argyropus	125	6	0	0
An. crawfordi	7	1	0	0
An. nigerrimus	63	60	4[a]	9[a]
An. nitidus	8	1	0	0
An. paraliae	59	13	5[b]	6[b]
An. pursati	89	67	8[c]	11[c]
An. sinensis	25	2	18[d]	15[d]

* Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

2) Mating ability of adult mosquitoes in 10, 20, 30 and 40 cubic cm cages at DRS of 7.2

The comparative mean insemination rates between stenogamous and eurygamous species demonstrated a statistically significant difference ($p < 0.05$) for all cage sizes (Table 3.7). Nonetheless, *An. peditaeniatus*, which had a high insemination rate, showed no statistically significant difference among cage sizes ($p > 0.05$).

Sperm density in the spermathecae of inseminated female mosquitoes of the eight species was based on grading, using various cage sizes at a DRS of 7.2, which are shown in Table 3.8-3.11. More than 50% of inseminated-female *An. peditaeniatus* showed high sperm density (3+ and 4+) in spermathecae in all cage sizes. Low sperm density (1+) was observed in the other seven species. The comparative frequency of sperm density, which was high at 3+ and 4+ between *An. peditaeniatus* and the four or five species (at 20 cubic cm cage), demonstrated a statistically significant difference ($p < 0.05$).

Table 3.7 Insemination rates of the eight species of the Hyrcanus Group using various cage sizes (10, 20, 30 and 40 cubic cm cages) at DRS of 7.2

Mosquito species	Mean insemination rate*		
	10 x 10 x 10 cm**	**20 x 20 x 20 cm****	**30 x 30 x 30 cm****
An. peditaeniatus	70.0	97.0	78.5
An. argyropus	37.5[a]	52.0[a]	69.5[a]
An. crawfordi	0[b]	0[b]	0.5[b]
An. nigerrimus	40.5[c]	79.0[c]	58.0[c]
An. nitidus	0[d]	4.0[d]	1.5[d]
An. paraliae	34.0[e]	44.0[e]	35.0[e]
An. pursati	61.0[f]	80.0[f]	40.5[f]
An. sinensis	47.5[g]	46.0[g]	37.0[g]

*Dissected 200 spermathecae/species/cage

**Chi-square test: a, b, c, d, e, f, g vs. *An. peditaeniatus* ($p < 0.05$)

Table 3.8 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (10 cubic cm cage, DRS = 7.2)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	2+	3+	4+
An. peditaeniatus	15	5	25	95
An. argyropus	65	10	0	0
An. crawfordi	0	0	0	0
An. nigerrimus	44	14	14[a]	9[a]
An. nitidus	0	0	0	0
An. paraliae	52	10	0	4[b]
An. pursati	100	10	0	12[c]
An. sinensis	10	10	10[b]	65[d]

*Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

Table 3.9 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (20 cubic cm cage, DRS = 7.2)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	2+	3+	4+
An. peditaeniatus	12	4	22	156
An. argyropus	104	0	0	0
An. crawfordi	0	0	0	0
An. nigerrimus	100	4	4[a]	50[a]
An. nitidus	6	1	1[b]	0
An. paraliae	40	6	6[c]	36[b]
An. pursati	92	22	8[d]	38[c]
An. sinensis	62	6	6[e]	18[d]

*Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d, e *vs. An. peditaeniatus* ($p < 0.05$)

Table 3.10 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (30 cubic cm cage, DRS = 7.2)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	2+	3+	4+
An. peditaeniatus	12	11	20	114
An. argyropus	138	1	0	0
An. crawfordi	1	0	0	0
An. nigerrimus	66	22	8[a]	20[a]
An. nitidus	2	0	1[b]	0
An. paraliae	36	5	11[c]	18[b]
An. pursati	68	5	0	8[c]
An. sinensis	19	6	13[d]	36[d]

*Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

Table 3.11 Frequency of inseminated grade based on sperm density in spermathecae of female mosquitoes of the eight species (40 cubic cm cage, DRS = 7.2)

Mosquito Species*	Grading of sperm density in female spermathecae**			
	1+	2+	3+	4+
An. peditaeniatus	18	38	42	80
An. argyropus	82	0	0	0
An. crawfordi	1	4	0	0
An. nigerrimus	46	24	7^{a}	7^{a}
An. nitidus	17	1	0	0
An. paraliae	60	0	5^{b}	5^{b}
An. pursati	85	4	0	4^{c}
An. sinensis	44	0	2^{c}	2^{d}

*Dissected 200 spermathecae/species

** Chi-square test: a, b, c, d *vs. An. peditaeniatus* ($p < 0.05$)

3.4.2 Measurements and large sensilla coeloconica counts under light and scanning electron microscopy (SEM)

1) Measurements of wings in adult females and males

The length and width of wings in females and males of the eight species of the Hyrcanus Group were measured in order to compute a ratio (length/width). Comparisons of mean wing ratios of females of the stenogamous *An. peditaeniatus* and the seven eurygamous species were statistically significant difference ($p < 0.05$), except for *An. nigerrimus*, *An. nitidus* and *An. pursati* ($p > 0.05$). Additionally, comparative mean wing ratios of males of the eight species were statistically significant difference ($p < 0.05$) (Table 3.12).

Table 3.12 Comparisons of mean wing ratios of females and males of the eight species of the Hyrcanus Group (30 females or males/species, n = 60)

Mosquito species		Measurements of wings of females (range)				Measurement of wings of males (range)		
		Length (µm)	Width (µm)	Mean wing ratio*		Length (µm)	Width (µm)	Mean wing ratio*
An. peditaeniatus	Right	1,676.89 ± 63.35	462.22 ± 21.15		Right	1,594.04 ± 48.17	426.49 ± 12.76	
		(1,577.23-1,820.49)	(412.21-513.83)	3.63		(1,482.64-1,728.09)	(402.61-471.53)	3.75
	Left	1,677.17 ± 63.75	462.16 ± 20.84		Left	1,597.87 ± 48.89	425.99 ± 12.75	
		(1,577.79-1,820.66)	(415.82-512.11)			(1,483.24-1,729.46)	(401.57-470.30)	
An. argyropus	Right	1,544.01 ± 83.24	406.94 ± 21.93		Right	1,570.52 ± 56.24	393.13 ± 12.98	
		(1,281.50-1,682.42)	(348.70-449.91)	3.81[a]		(1,457.86-1,683.84)	(372.61-422.80)	4.00[h]
	Left	1,556.37 ± 85.27	406.78 ± 21.69		Left	1,571.88 ± 54.28	393.11 ± 13.17	
		(1,285.59-1,682.98)	(351.11-449.00)			(1,451.24-1,681.56)	(370.86-426.61)	
An. crawfordi	Right	1,757.79 ± 52.51	461.99 ± 15.68		Right	1,674.92 ± 70.65	415.68 ± 15.06	
		(1,667.70-1,863.26)	(431.55-490.29)	3.81[b]		(1,504.72-1,850.72)	(371.58-437.78)	4.04[i]
	Left	1,757.36 ± 51.72	460.20 ± 14.70		Left	1,674.09 ± 70.69	414.86 ± 15.44	
		(1,665.63-1,856.58)	(430.10-482.59)			(1,501.79-1,849.61)	(371.01-440.53)	
An. nigerrimus	Right	1,539.24 ± 69.02	434.00 ± 21.55		Right	1,524.94 ± 52.78	384.27 ± 10.81	
		(1,377.28-1,650.21)	(362.86-470.50)	3.55[c]		(1,453.34-1,683.51)	(354.21-404.76)	3.97[j]
	Left	1,538.67 ± 68.58	433.75 ± 20.95		Left	1,525.13 ± 54.62	384.86 ± 10.80	
		(1,387.92-1,650.63)	(363.60-470.50)			(1,452.59-1,680.61)	(358.71-408.08)	

Table 3.12 (continued)

Mosquito species		Measurements of wings of females (range)				Measurement of wings of males (range)		
		Length (µm)	Width (µm)	Mean wing ratio*		Length (µm)	Width (µm)	Mean wing ratio*
An. nitidus	Right	1,539.33 ± 77.41	451.37 ± 23.97		Right	1,611.04 ± 45.61	358.95 ± 15.82	
		(1,378.83-1,676.56)	(381.13-462.01)	3.71[d]		(1,521.84-1,707.28)	(353.13-411.92)	4.17[k]
	Left	1,539.60 ± 76.95	414.93 ± 22.88		Left	1610.62 ± 45.16	386.06 ± 15.20	
		(1,379.23-1,675.89)	(381.09-462.63)			(1,523.76-1,704.20)	(354.54-410.12)	
An. paraliae	Right	1,716.26 ± 55.43	451.32 ± 18.64		Right	1,663.67 ± 48.83	397.85 ± 13.82	
		(1,620.99-1,825.03)	(409.89-486.01)	3.81[e]		(1,556.00-1,745.86)	(360.74-425.83)	4.19[l]
	Left	1,715.10 ± 53.99	450.64 ± 18.73		Left	1,662.94 ± 48.19	397.15 ± 13.70	
		(1,560.67-1,823.87)	(407.68-484.21)			(1,559.49-1,743.82)	(359.76-422.63)	
An. pursati	Right	1,455.64 ± 53.95	398.20 ± 13.85		Right	1,424.06 ± 63.55	355.66 ± 17.03	
		(1,355.55-1,549.94)	(358.12-422.51)	3.66[f]		(1,248.31-1,585.17)	(321.80-389.42)	4.02[m]
	Left	1,455.30 ± 55.11	398.65 ± 12.97		Left	1,424.35 ± 65.77	352.23 ± 23.25	
		(1,343.30-1,548.63)	(370.92-423.71)			(1,254.56-1,586.07)	(264.19-388.84)	
An. sinensis	Right	1,756.11 ± 75.51	468.33 ± 20.49		Right	1,626.20 ± 74.16	402.39 ± 17.06	
		(1,617.83-1,911.86)	(436.35-519.06)	3.75[g]		(1,453.31-1,772.68)	(373.80-434.83)	4.05[n]
	Left	1,757.11 ± 74.59	468.06 ± 20.37		Left	1,626.14 ± 74.02	401.90 ± 17.06	
		(1,618.31-1,911.33)	(435.02-517.08)			(1,456.65-1,769.68)	(373.43-433.44)	

*Tukey's HSD test: a, b, e, g, h, i, j, k, l, m, n vs. *An. peditaeniatus*, $p < 0.05$; c, d, f vs. *An. peditaeniatus*, $p > 0.05$

2) Measurements of maxillary palpomeres of females

The results of comparisons of mean palpomeres ratios in females of the stenogamous *An. peditaeniatus* and the seven eurygamous species were statistically significant difference ($p < 0.05$) (Table 3.13).

Table 3.13 Comparisons of the mean palpomeres ratios of females of the eight species of the Hyrcanus Group (30 females/species, $n = 60$)

Mosquito species	Mean length of palpomeres (range)			Mean palpomeres ratio* (range)
	Length of palpomere 5 (µm)	Length of palpomere 4 (µm)	Length of palpomere 3 (µm)	
An. peditaeniatus	241.17 ± 15.66 (211.63-263.82)	407.14 ± 23.36 (355.70-445.97)	654.09 ± 33.17 (588.89-730.01)	0.90 ± 0.05 (0.81-1.00)
An. argyropus	209.79 ± 23.06 (172.33-253.31)	335.59 ± 33.86 (259.87-386.26)	525.36 ± 41.03 (434.07-598.88)	1.04 ± 0.08 (0.92-1.27)[a]
An. crawfordi	216.67 ± 20.01 (179.43-279.36)	347.88 ± 27.16 (290.55-402.71)	740.77 ± 32.12 (686.40-812.34)	0.99 ± 0.06 (0.85-1.09)[b]
An. nigerrimus	189.42 ± 17.18 (154.08-224.54)	330.03 ± 27.77 (268.27-388.07)	579.99 ± 29.72 (514.26-640.13)	0.75 ± 0.06 (0.49-0.84)[c]
An. nitidus	201.60 ± 17.30 (174.62-234.18)	329.78 ± 31.12 (248.09-371.48)	593.07 ± 42.18 (507.26-659.56)	0.88 ± 0.05 (0.75-0.96)[d]
An. paraliae	240.92 ± 19.37 (199.52-272.22)	384.22 ± 24.42 (341.02-421.47)	713.43 ± 34.37 (648.87-784.68)	0.86 ± 0.04 (0.77-0.96)[e]
An. pursati	188.99 ± 14.12 (165.65-217.92)	275.09 ± 25.19 (210.15-327.66)	541.04 ± 25.54 (490.78-589.98)	0.90 ± 0.06 (0.77-1.04)[f]
An. sinensis	252.74 ± 72.32 (200.28-551.89)	381.61 ± 38.04 (307.87-488.98)	679.53 ± 87.05 (311.31-771.51)	0.88 ± 0.04 (0.77-0.95)[g]

*Tukey's HSD test: a, b, c, d, e, f, g vs. *An. peditaeniatus*, $p < 0.05$

3) Number and form of large sensilla coeloconica on antennae of females

Attempts were made to count the number of large sensilla coeloconica on the antennal flagellum of females of the eight species of the Hyrcanus Group. However, it was not possible to count individual sensilla of *An. argyropus* and *An. peditaeniatus* as they are arrayed in a cluster on the flagellomeres (Figure 3.3). This was observed more clearly under SEM (Figure 3.4). SEM observations revealed that the groups of 4-7 large sensilla coeloconica occur mainly on flagellomeres 3-7 of *An. peditaeniatus*. *Anopheles argyropus* also bears a cluster of large sensilla coeloconica on flagellomeres 3-7. However, the large sensilla coeloconica of *An. peditaeniatus* are located as a group in deeper sacculi than in *An. argyropus*. In contrast, the other six species have sensilla that occur as a single pit on their flagellomeres (Figures 3.5 and 3.6, Table 3.14). In addition, three types of sensilla also are found on the antennae of these species, i.e., sensilla chaetica (bristles), sensilla trichodea (hairs) and sensilla basiconica (grooved pegs) (Figures 3.4 and 3.6).

The mean number of large sensilla coeloconica on the antennal flagellum of females was calculated from 30 individuals of each of the six species; the means are shown in Table 3.14. The number of large sensilla coeloconica per antennal flagellomere ranged from 0-6, 0-7, 0-6, 0-9, 0-6 and 0-8 in *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. pursati* and *An. sinensis*, respectively. The total number of large sensilla coeloconica per antennae ranged between 45-63, 56-78, 50-69, 52-89, 51-69 and 57-88 in *An. crawfordi*, *An. nigerrimus*, *An. nitidus*, *An. paraliae*, *An. pursati* and *An. sinensis*, respectively. The mean numbers of large sensilla coeloconica per antennae of the six species were not statistically significant difference ($p > 0.05$). It is interesting to note that no large sensilla coeloconica are present on the terminal flagellomere (13) of *An. nitidus* (Table 3.14).

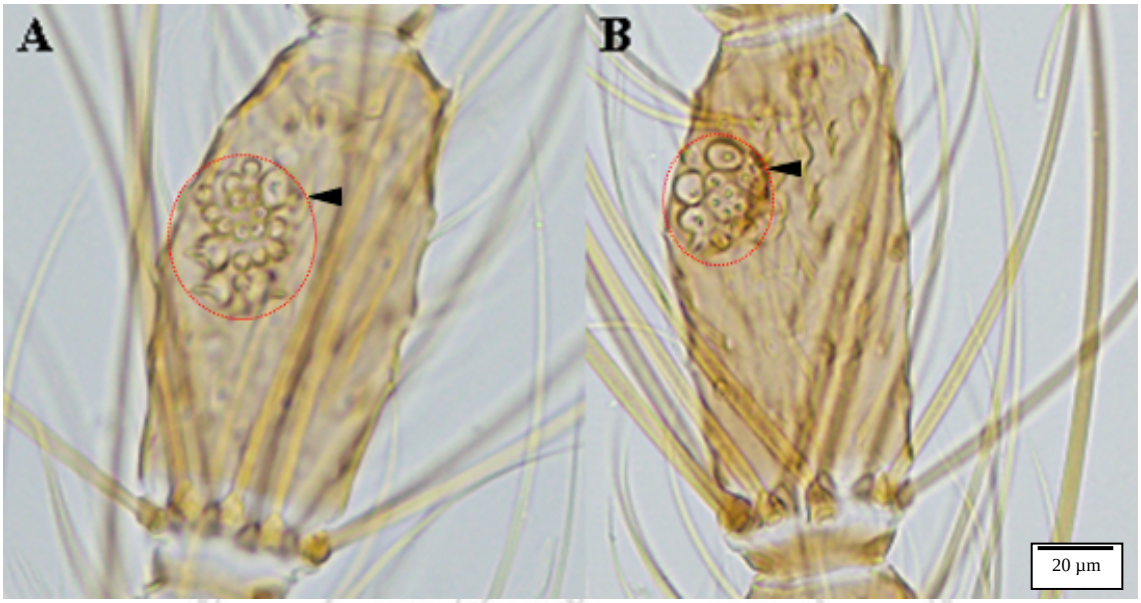

Figure 3.3 The cluster of large sensilla coeloconica located in a sunken depression (sacculus) on the 5th antennal flagellomere of *An. argyropus* (A) and *An. peditaeniatus* (B), photographed under a light microscope. Scale bar: 0.02. mm

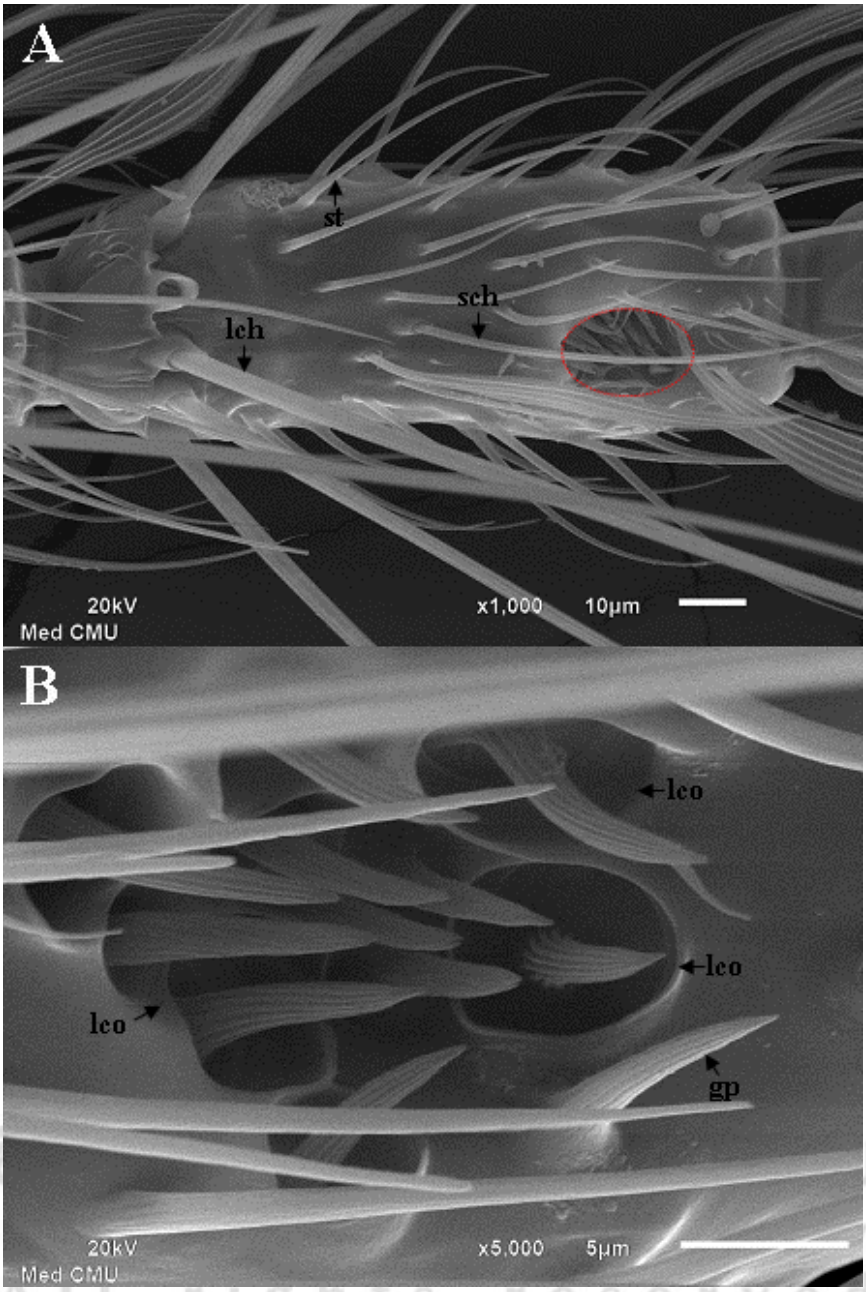

Figure 3.4 Scanning electron micrographs of the 5th antennal flagellomere of *An. peditaeniatus*: (A) sacculus (circle) containing large sensilla coeloconica (1,000x); (B) higher magnification of the sacculus with its large sensilla coeloconica (5,000x). The different types of sensilla shown in the micrographs occur on most flagellomeres: lch, large chaetica; sch, small chaetica; st, sharp trichoid; lco, large coeloconica and gp, grooved peg

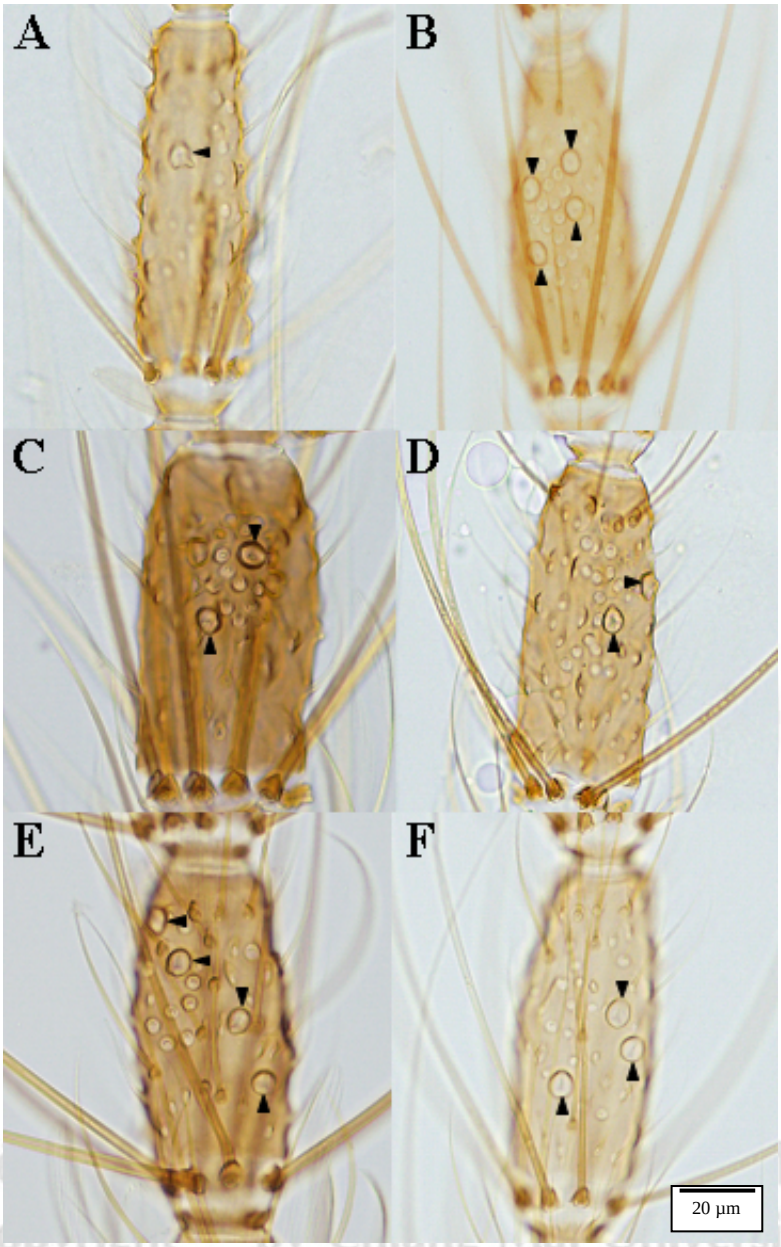

Figure 3.5 The distribution of large sensilla coeloconica on the 5th antennal flagellomere of six species of the Hyrcanus Group, photographed under a light microscope: A, *An. crawfordi*; B, *An. nitidus*; C, *An. nigerrimus*; D, *An. paraliae*; E, *An. pursati* and F, *An. sinensis*. Scale bar: 0.02 mm

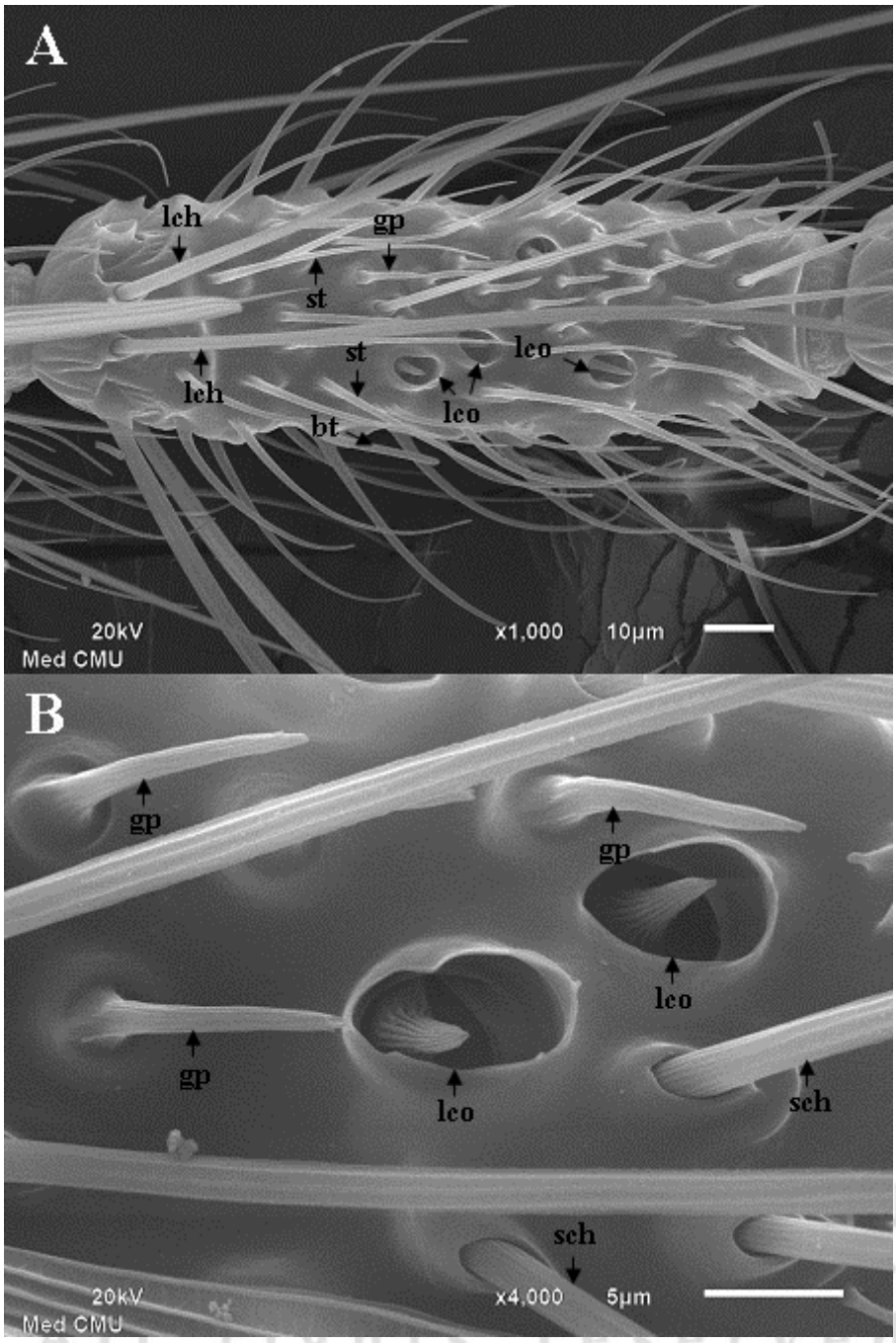

Figure 3.6 Scanning electron micrographs of the 5th antennal segment of *An. paraliae*. A, showing large sensilla coeloconica (x 1,000) and B, a higher magnification of large sensilla coeloconica (x 4,000). The different types of sensilla shown in the micrographs occur on most flagellomeres: lch, large chaetica; sch, small chaetica; st, sharp trichoid; bt, blunt trichoid; lco, large coeloconica and gp, grooved peg

Table 3.14 Mean distributions of sensilla coeloconica on the 13 antennal flagellomeres of females of the six species of the Hyrcanus Group (30 females/species, n = 60)

Antennal segment	Mosquito species							
	CF* (range)	NG-* (range)	NT* (range)	PR* (range)	PS* (range)	SN* (range)	PD (range)	AG (range)
1	2.35 ± 0.71 (1-4)	3.15 ± 0.69 (2-6)	2.82 ± 0.68 (1-4)	5.20 ± 1.16 (1-7)	1.93 ± 0.52 (1-3)	4.08 ± 0.85 (2-7)	NA	NA
2	3.45 ± 0.87 (2-5)	4.52 ± 1.00 (3-7)	3.67 ± 0.86 (3-6)	6.23 ± 1.03 (4-9)	3.88 ± 0.85 (2-5)	5.12 ± 1.28 (3-8)	NA	NA
3	3.18 ± 0.85 (1-5)	5.17 ± 0.98 (3-7)	4.68 ± 0.70 (3-6)	4.98 ± 1.10 (2-7)	3.80 ± 0.82 (3-6)	5.92 ± 1.18 (3-8)	NA	NA
4	2.42 ± 0.87 (1-5)	4.32 ± 0.97 (2-7)	3.83 ± 0.81 (2-6)	3.65 ± 1.21 (2-6)	4.02 ± 0.95 (2-6)	4.17 ± 0.76 (3-7)	NA	NA
5	2.10 ± 0.63 (1-3)	3.02 ± 0.95 (1-5)	3.08 ± 0.85 (1-5)	2.93 ± 1.01 (1-5)	2.48 ± 0.79 (1-5)	3.22 ± 0.83 (2-5)	NA	NA
6	1.83 ± 0.64 (1-3)	2.68 ± 0.83 (1-4)	2.18 ± 0.73 (1-4)	2.77 ± 0.89 (1-5)	2.57 ± 0.87 (1-5)	2.92 ± 0.79 (1-5)	NA	NA
7	1.70 ± 0.65 (0-3)	2.23 ± 0.81 (1-4)	1.53 ± 0.62 (1-3)	2.37 ± 0.78 (1-4)	1.37 ± 0.64 (0-3)	2.31 ± 0.85 (1-4)	NA	NA
8	1.53 ± 0.60 (1-3)	1.68 ± 0.67 (0-3)	1.23 ± 0.43 (1-2)	1.85 ± 0.80 (1-3)	1.42 ± 0.67 (1-3)	2.10 ± 0.80 (0-4)	NA	NA
9	0.90 ± 0.48 (0-2)	1.03 ± 0.37 (0-2)	0.87 ± 0.39 (0-2)	1.25 ± 0.65 (0-3)	0.98 ± 0.23 (0-2)	0.98 ± 0.43 (0-2)	NA	NA
10	1.02 ± 0.34 (0-2)	0.95 ± 0.39 (0-2)	0.97 ± 0.32 (0-1)	1.07 ± 0.45 (0-2)	1.08 ± 0.38 (0-2)	1.10 ± 0.48 (0-2)	NA	NA
11	1.62 ± 0.72 (1-3)	1.30 ± 0.46 (0-2)	1.28 ± 0.56 (0-2)	1.58 ± 0.59 (0-2)	1.53 ± 0.50 (1-2)	1.45 ± 0.65 (0-3)	NA	NA
12	3.73 ± 1.01 (2-6)	3.02 ± 0.62 (2-5)	2.58 ± 0.53 (2-4)	3.00 ± 0.74 (1-6)	2.75 ± 0.80 (1-5)	3.07 ± 0.80 (1-5)	NA	NA
13	0.02 ± 0.13 (0-1)	0.03 ± 0.18 (0-1)	0.00 ± 0.00 (0)	0.67 ± 0.75 (0-2)	0.03 ± 0.26 (0-1)	0.03 ± 0.18 (0-2)	NA	NA
Total (range)	25.78 ± 8.48 (45-63)	33.10 ± 8.92 (56-78)	28.73 ± 7.46 (50-69)	37.55 ± 11.15 (52-89)	27.85 ± 8.26 (51-69)	36.47 ± 9.872 (57-88)	NA	NA

CF = *An. crawfordi*; NG = *An. nigerrimus*; NT = *An. nitidus* ; PR = *An. paraliae*; PS = *An. pursati*; SN = *An. sinensis*; PD = *An. peditaeniatus*; AG = *An. argyropus*

NA = not available

*p > 0.05, Tukey's HSD test

4) Measurements of male genitalia

Measurements of male genital structures in the eight species of the Hyrcanus Group are shown in Table 3.15. Comparisons of the results of statistical analyses between stenogamous and eurygamous species was given in Table 3.16. The results show that all measurements of the male genitalia of *An. peditaeniatus* are statistically significant difference from those of the other species ($p < 0.05$). For example, the aedeagus length of *An. peditaeniatus* is significantly shorter than the aedeagus of *An. argyropus*, *An. crawfordi*, *An. nitidus* and *An. sinensis* ($p < 0.05$). Remarkably, the gonostylus length of *An. peditaeniatus* is significantly shorter than the gonostylus of the seven eurygamous species ($p < 0.05$) (Table 3.16).

Table 3.15 Comparisons of measurements (length and width in microns) of male genital structures in the eight species of the Hyrcanus Group (30 males/species)

Character	Mosquito species							
	PD (range)	**AG** (range)	**CF** (range)	**NG** (range)	**NT** (range)	**PR** (range)	**PS** (range)	**SN** (range)
Length of aedeagus	37.47 ± 8.14 (25.13-55.60)	40.52 ± 6.39 (30.24-50.28)	52.64 ± 4.66 (40.89-59.56)	46.34 ± 4.30 (38.77-55.34)	42.31 ± 4.93 (35.06-52.13)	50.63 ± 3.43 (43.17-57.41)	40.05 ± 3.65 (35.65-51.95)	57.94 ± 3.60 (50.66-65.47)
Width of aedeagus	33.53 ± 4.16 (25.09-41.43)	36.19 ± 5.43 (26.2-51.29)	37.19 ± 5.37 (26.95-50.60)	36.10 ± 4.22 (28.54-42.63)	33.13 ± 3.60 (26.85-40.95)	32.97 ± 3.34 (28.48-44.58)	30.74 ± 3.38 (24.09-36.11)	37.32 ± 4.38 (31.72-48.53)
Length between base of aedeagus and origin of gonocoxite (right)	47.10 ± 5.77 (38.78-63.36)	44.45 ± 4.98 (35.43-54.93)	49.01 ± 7.68 (31.74-63.86)	44.18 ± 5.89 (31.13-56.32)	45.47 ± 5.09 (31.85-53.73)	45.50 ± 5.47 (31.49-55.91)	45.75 ± 5.89 (38.53-60.95)	52.42 ± 4.18 (43.56-61.78)
Length between base of aedeagus and origin of gonocoxite (left)	47.50 ± 5.01 (39.82-61.77)	44.88 ± 4.70 (36.21-53.94)	48.73 ± 6.87 (32.39-63.10)	44.62 ± 5.29 (31.52-55.32)	45.93 ± 4.94 (31.13-53.69)	45.35 ± 5.37 (33.71-54.20)	45.85 ± 5.80 (39.39-61.68)	52.31 ± 4.14 (44.08-61.87)
Width of gonocoxite at origin of parabasal seta (right)	78.42 ± 7.37 (65.96-92.09)	80.64 ± 4.40 (67.13-88.75)	83.13 ± 3.93 (73.60-90.97)	86.79 ± 5.41 (70.79-95.90)	83.04 ± 5.86 (66.29-91.74)	84.08 ± 3.49 (77.09-89.75)	76.42 ± 6.34 (68.20-94.90)	95.39 ± 5.06 (87.26-105.43)
Width of gonocoxite at origin of parabasal seta (left)	78.60 ± 7.43 (66.26-93.80)	80.27 ± 4.48 (65.99-90.79)	83.22 ± 3.85 (73.80-90.55)	86.07 ± 5.23 (70.48-93.17)	83.96 ± 6.00 (67.69-93.87)	83.74 ± 3.04 (78.29-89.40)	76.25 ± 5.91 (68.30-93.31)	95.10 ± 4.96 (87.19-105.21)

Table 3.15 (continued)

Character	Mosquito species							
	PD (range)	**AG** (range)	**CF** (range)	**NG** (range)	**NT** (range)	**PR** (range)	**PS** (range)	**SN** (range)
Length of gonocoxite (right)	135.38 ± 9.52 (117.42-150.71)	134.64 ± 8.38 (121.00-158.87	135.30 ± 6.44 (120.61-146.48)	148.57 ± 11.15 (123.10-165.83)	145.85 ± 6.34 (135.23-160.90)	149.60 ± 6.61 (136.62-159.42)	135.48 ± 6.94 (126.11-150.74)	155.35 ± 9.96 (137.62-182.04)
Length of gonocoxite (left)	135.35 ± 9.30 (118.54-150.99)	135.30 ± 9.10 (121.22-157.54)	135.29 ± 6.14 (122.71-145.53)	149.01 ± 10.79 (124.15-164.87)	145.42 ± 6.14 (136.59-159.93)	149.48 ± 5.92 (138.70-157.41)	135.46 ± 6.85 (125.70-152.87)	191.46 ± 7.08 (176.44-203.08)
Length of gonostylus (right)	160.58 ± 8.85 (146.89-174.59)	167.99 ± 6.41 (158.75-190.81)	172.02 ± 5.71 (159.00-181.93)	175.84 ± 8.18 (150.16-191.19)	177.47 ± 6.75 (164.89-192.20)	180.86 ± 6.21 (168.83-193.76)	167.08 ± 6.65 (153.15-178.10)	191.46 ± 7.08 (176.44-203.08)
Length of gonostylus (left)	160.56 ± 8.84 (146.24-175.32)	167.53 ± 4.44 (158.82-177.75)	172.30 ± 5.35 (162.87-181.99)	176.06 ± 8.02 (153.58-191.86)	177.10 ± 6.59 (165.59-192.01)	180.72 ± 5.78 (167.11-192.24)	166.77 ± 6.28 (155.46-178.45)	191.74 ± 7.21 (177.53-206.80)
Length of gonocoxite + gonostylus (right)	295.96 ± 16.66 (270-322)	302.63 ± 11.36 (285-329)	307.32 ± 10.14 (286-325)	324.41 ± 15.86 (273-352)	323.31 ± 9.89 (303-343)	330.45 ± 10.47 (308-350)	302.56 ± 12.54 (284-326)	346.96 ± 15.36 (315-379)
Length of gonocoxite + gonostylus (left)	295.91 ± 16.42 (268-326)	302.82 ± 10.61 (284-326)	307.59 ± 9.42 (290-324)	325.07 ± 15.52 (278-351)	322.52 ± 9.33 (304-343)	330.20 ± 9.87 (310-348)	302.22 ± 12.07 (286-328)	347.09 ± 15.30 (315-376)

PD = *An. peditaeniatus*; AG = *An. argyropus*; CF = *An. crawfordi*; NG = *An. nigerrimus*; PR = *An. paraliae*; PS = *An. pursati*; SN = *An. sinensis*

Table 3.16 Comparisons of the results of statistical analyses of male genital measurements for the eight species of the Hyrcanus Group

Character	**Mosquito species**		***p*-value**
Length of aedeagus	*An. peditaeniatus* vs	*An. argyropus*	0.000*
		An. crawfordi	0.001*
		An. nigerrimus	0.884
		An. nitidus	0.000*
		An. paraliae	0.477
		An. pursati	1.000
		An. sinensis	0.000*
Width of aedeagus	*An. peditaeniatus* vs	*An. argyropus*	0.252
		An. crawfordi	0.025*
		An. nigerrimus	0.294
		An. nitidus	1.000
		An. paraliae	1.000
		An. pursati	0.194
		An. sinensis	0.017*
Length between base of aedeagus and origin of gonocoxite (right)	*An. peditaeniatus* vs	*An. argyropus*	0.620
		An. crawfordi	0.889
		An. nigerrimus	0.493
		An. nitidus	0.958
		An. paraliae	0.955
		An. pursati	0.984
		An. sinensis	0.009*
Length between base of aedeagus and origin of gonocoxite (left)	*An. peditaeniatus* vs	*An. argyropus*	0.546
		An. crawfordi	0.987
		An. nigerrimus	0.419
		An. nitidus	0.962
		An. paraliae	0.733
		An. pursati	0.930
		An. sinensis	0.013*

Table 3.16 (continued)

Character	Mosquito species		*p*-value
Width of gonocoxite at origin of parabasal seta (right)	*An. peditaeniatus* vs	*An. argyropus*	0.809
		An. crawfordi	0.000*
		An. nigerrimus	0.000*
		An. nitidus	0.014*
		An. paraliae	0.015*
		An. pursati	0.876
		An. sinensis	0.000*
Width of gonocoxite at origin of parabasal seta (left)	*An. peditaeniatus* vs	*An. argyropus*	0.921
		An. crawfordi	0.017*
		An. nigerrimus	0.000*
		An. nitidus	0.001*
		An. paraliae	0.016
		An. pursati	0.668
		An. sinensis	0.000*
Length of gonocoxite (right)	*An. peditaeniatus* vs	*An. argyropus*	1.000
		An. crawfordi	1.000
		An. nigerrimus	0.000*
		An. nitidus	0.000*
		An. paraliae	0.000*
		An. pursati	1.000
		An. sinensis	0.000*
Length of gonocoxite (left)	*An. peditaeniatus* vs	*An. argyropus*	1.000
		An. crawfordi	1.000
		An. nigerrimus	0.000*
		An. nitidus	0.000*
		An. paraliae	0.000*
		An. pursati	1.000
		An. sinensis	0.000*

Table 3.16 (continued)

Character	Mosquito species		*p*-value
Length of gonostylus (right)	*An. peditaeniatus* vs	*An. argyropus*	0.002*
		An. crawfordi	0.000*
		An. nigerrimus	0.000*
		An. nitidus	0.000*
		An. paraliae	0.000*
		An. pursati	0.010*
		An. sinensis	0.000*
Length of gonostylus (left)	*An. peditaeniatus* vs	*An. argyropus*	0.002*
		An. crawfordi	0.000*
		An. nigerrimus	0.000*
		An. nitidus	0.000*
		An. paraliae	0.000*
		An. pursati	0.010*
		An. sinensis	0.000*
Ratio length and width of gonocoxite	*An. peditaeniatus* vs	*An. argyropus*	0.646
		An. crawfordi	0.013*
		An. nigerrimus	1.000
		An. nitidus	0.997
		An. paraliae	0.646
		An. pursati	0.739
		An. sinensis	0.018*
Ratio length and width of gonocoxite + gonostylus	*An. peditaeniatus* vs	*An. argyropus*	0.443
		An. crawfordi	0.013*
		An. nigerrimus	0.000*
		An. nitidus	0.000*
		An. paraliae	0.000*
		An. pursati	0.512
		An. sinensis	0.000*

*Significantly different ($p < 0.05$, Tukey's HSD test)

5) Clasper movement and duration of mating

The frequency of clasper movement in the stenogamous *An. peditaeniatus* was lower than that in the seven eurygamous species, with statistical significance ($p < 0.05$). Similarly, the mating time of *An. peditaeniatus* was shorter than that in the other species, with statistical significance ($p < 0.05$), except for *An. argyropus* (Table 3.17).

Table 3.17 Comparative measurements of mating time (duration in seconds) and frequency of clasper movement per copulation in the eight species of the Hyrcanus Group ($n = 30$)

Mosquito species	Frequency of clasper movement (range)*	Mating time (range)*
An. peditaeniatus	5.62 ± 1.05 (3.5-8.0)	2.9 ± 0.4 (1.5-3.5)
An. argyropus	6.68 ± 0.81 (5.5-8.5)[a]	2.8 ± 0.4 (2.5-4.0)[h]
An. crawfordi	7.83 ± 1.28 (5.0-10.5)[b]	5.4 ± 0.6 (4.0-7.0)[i]
An. paraliae	8.07 ± 1.03 (6.0-10.0)[c]	3.9 ± 0.4 (3.5-5.0)[j]
An. pursati	7.22 ± 1.22 (4.5-10.0)[d]	3.6 ± 0.5 (3-4.5.0)[k]
An. nigerrimus	7.52 ± 1.52 (5.0-11.5)[e]	4.1 ± 0.8 (2.5-6.0)[l]
An. nitidus	7.87 ± 1.11 (5.5-10.0)[f]	3.7 ± 0.5 (3.0-5.0)[m]
An. sinensis	8.70 ± 1.40 (7.0-13.0)[g]	4.6 ± 0.7 (4.0-6.0)[n]

*Tukey's HSD test: a, b, c, d, e, f, g, i, j, k, l, m, n vs. *An. peditaeniatus*, $p < 0.05$;
h vs. *An. peditaeniatus*, $p > 0.05$