CHAPTER 4

RESULTS AND DISCUSSION

PAPER 1: Analysis of female salivary gland proteins of the *Anopheles barbirostris* complex (Diptera: Culicidae) in Thailand

RESULTS

Mosquito collection and identification of mosquito forms and species

Wild-caught, fully engorged females of the *An. barbirostris* complex were collected in eight provinces in Thailand (Fig. 1 and Table 1). A total of 11 isoline colonies were successfully established. Results of mosquito forms and species are shown in Figure 2 and Table 1.

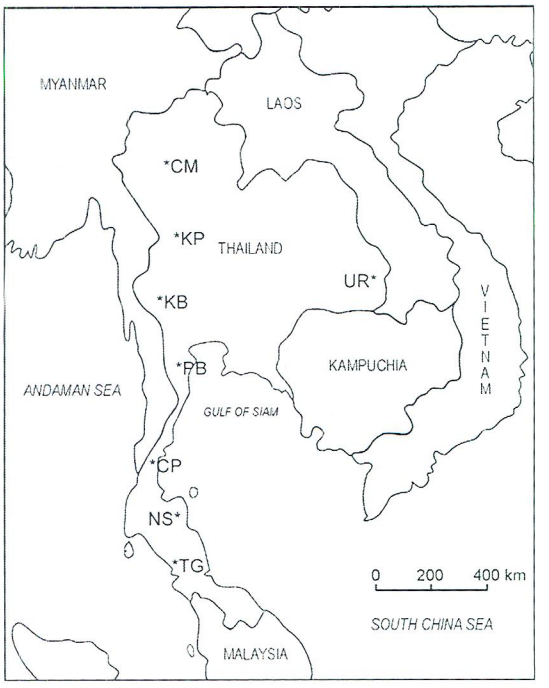

Fig. 1 Collection sites (*) for the *An. barbirostris/campestris* group in Thailand. CM: Chiang Mai; KP: Kamphaeng Phet; KB: Kanchanaburi; UR: Ubon Ratchathani; PB: Phetchaburi; CP: Chumphon; NS: Nakhon Si Thammarat; TG: Trang.

Table 1 Localities where the *An. barbirostris* complex were collected, including geographic coordinates, strain number, code and karyotypic form, length of ITS2, and species

Locality (Geographic coordinates)	Strain number and code[a]	Karyotypic form (X,Y)	Length of ITS2 (base pair)	Species
Chiang Mai (18°47′N, 98°59′E)	aCMA1	A (X_2,Y_1)	1,822	*An. barbirostris* A1
	aCMA2	A (X_2,Y_1)	1,637	*An. barbirostris* A4
	hCME3	E (X_2,Y_5)	1,612	*An. campestris*-like
	hCME4	E (X_2,Y_5)	1,612	*An. campestris*-like
Kamphaeng Phet (16°28′N, 99°31′E)	aKPB1	B (X_2,Y_2)	1,612	*An. campestris*-like
Kanchanaburi (14°01′N, 99°32′E)	aKBA2	A (X_2,Y_1)	1,031	*An. barbirostris* A3
Ubon Ratchathani (15°15′N, 104°52′E)	aUBA7	A (X_2,Y_1)	1,822	*An. barbirostris* A1
Phetchaburi (13°09′N, 100°04′E)	aPBA3	A (X_2,Y_1)	1,678	*An. barbirostris* A2
Chumphon (10°29′N, 99°11′E)	aCPB4	B (X_1,Y_2)	1,822	*An. barbirostris* A1
Nakhon Si Thammarat (08°32′N, 99°57′E)	aNSD1	D (X_2,Y_4)	1,822	*An. barbirostris* A1
Trang (07°31′N, 99°37′E)	aTGA10	A (X_2,Y_1)	1,822	*An. barbirostris* A1

[a]h – human bait; a – animal bait

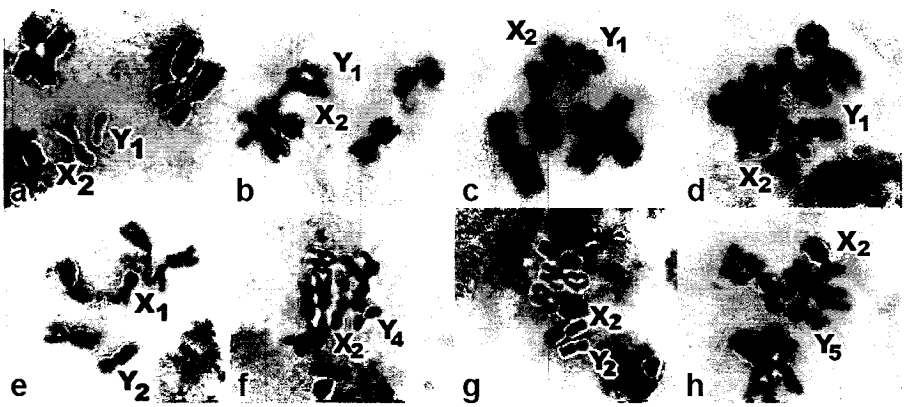

Fig. 2 Metaphase karyotypes of *An. barbirostris* complex. **(a)** species A1, Form A (X_2, Y_1: Chiang Mai); **(b)** species A2, Form A (X_2, Y_1: Phetchaburi); **(c)** species A3, Form A (X_2, Y_1: Kanchaburi); **(d)** species A4, Form A (X_2, Y_1: Chiang Mai); **(e)** species A1, Form B (X_1, Y_2: Chumphon); **(f)** species A1, Form D (X_2, Y_4: Nakhon Si Thammarat); **(g)** *An. campestris*-like, Form B (X_2, Y_2: Kamphaeng Phet); **(h)** *An. campestris*-like, Form E (X_2, Y_5: Chiang Mai).

Analysis of salivary gland proteins of the mosquitoes by SDS-PAGE and NanoLC-MS

Comparison of electrophoretic profiles of salivary glands obtained from female *An. barbirostris* species A1, A2, A3, A4, and *An. campestris*-like captured in eight provinces of Thailand is shown in Fig. 3. At least eight major and several minor protein bands were detected in the glands of each species. The variability in major proteins among species was observed in the 40-50 kilodalton (kDa), 32-37 kDa, and 10-18 kDa ranges. The electrophoretic profiles of salivary gland proteins differed among species but no difference was found within species or different cytogenetic forms except *An. barbirostris* species A4. Polymorphism of the electrophoretic protein profiles in species A4 was observed in the protein with molecular mass of 36 kDa (Fig. 4). The lowest major protein band of each species with molecular mass of about 10 to 13 kDa showed remarkably different relative mobility on SDS-polyacrylamide gels (Marker bands, Fig. 3). Therefore, they were excised, trypsin-digested and analyzed by NanoLC-MS. LC-MS data was searched against all known *Anopheles* sequences using Mascot with trypsin as the proteolytic enzyme. Results showed that the protein of some sibling species matched a gSG6 protein of *An. gambiae* and *An. freeborni* (Table 2).

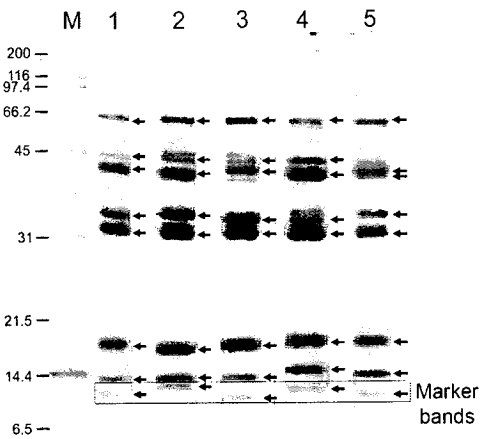

Fig. 3 Electrophoretic profiles of salivary glands obtained from female *An. barbirostris* complex. Proteins of one salivary gland pair were separated on 15% SDS-polyacrylamide gels and stained with Coomassie brilliant blue. Lane 1, *An. barbirostris* species A1; lane 2, species A2; lane 3, species A3; lane 4, species A4; lane 5, *An. campestris*-like; M, molecular mass makers (kDa). Arrows indicate major protein bands detected in samples of each species. Rectangle shows the marker band of each species.

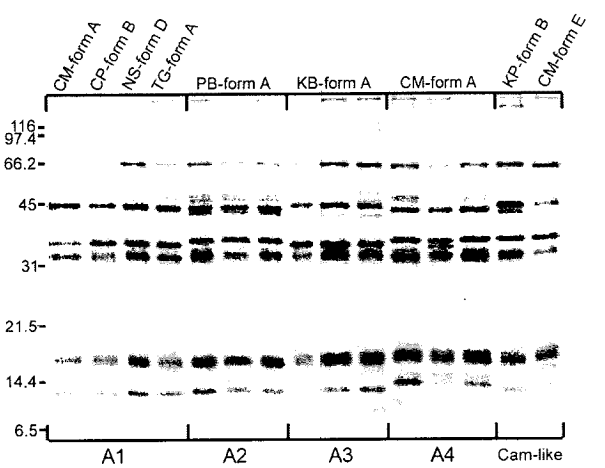

Fig. 4 Electrophoretic protein profiles of salivary glands obtained from female *An. barbirostris* species A1, A2, A3, A4, and *An. campestris*-like. Proteins of one pair of salivary gland were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Mosquitoes from CM: Chiang Mai; CP: Chumphon; NS: Nakhon Si Thammarat; TG: Trang; PB: Phetchaburi; KB: Kanchanaburi; KP: Kamphaeng Phet. Molecular mass markers are shown on the left in kilodalton.

Table 2 Results of the marker protein band of each species identified by NanoLC-MS

Species	Accession number	Protein score[b]	Description
An. barbirostris A1	gi|13537666	110	gSG6 protein [*An. gambiae*]
An. barbirostris A2	No match[a]	-	-
An. barbirostris A3	gi|229418592	119	gSG6 salivary protein [*An. freeborni*]
An. barbirostris A4	No match	-	-
An. campestris-like	gi|13537666	87	gSG6 protein [*An. gambiae*]

[a]No match to *Anopheles* sequences

[b]$P < 0.05$

Investigation of the proteins in each salivary gland lobes

Total proteins in the distal-lateral lobes and median lobes of the female salivary glands of the five sibling species were examined in CBB stained SDS-polyacrylamide gels (Fig. 5). The different morphological regions of the female salivary glands displayed distinct electrophoretic protein profiles. In each species, the major protein bands in the 32-37 kDa and 10-18 kDa ranges including the marker bands appeared predominantly in the distal region of the lateral lobe, while protein bands in the 40-50 kDa, 32-34 kDa and 13-15 kDa ranges were predominant in the medial lobe. The protein profiles of the proximal regions of the lateral lobes were not examined as they appeared similar to the profile of the male salivary glands in most mosquito species and represented proteins that involve sugar feeding (Marinotti et al. 1996; Moreira et al. 2001).

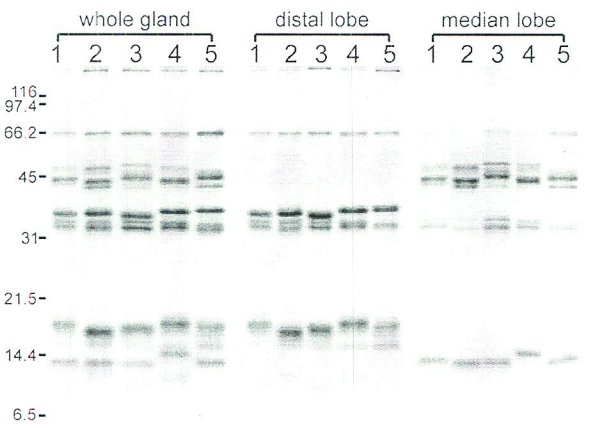

Fig. 5 Electrophoretic protein profiles of salivary glands obtained from female *An. barbirostris* species A1, A2, A3, A4, and *An. campestris*-like. Proteins of one pair of whole salivary gland or distal lobe or median lobe were separated on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. 1: *An. barbirostris* species A1; 2: species A2; 3: species A3; 4: species A4; 5: *An. campestris*-like. Molecular mass makers are shown on the left in kilodalton.

DISCUSSION

Thailand is an endemic area for malaria. A number of sibling species complexes of Anopheline mosquitoes are the human malaria vectors, for example, *An. dirus* and *An. minimus* (Rattanarithikul et al. 2006). Recently, *An. barbirostris* complex, i.e., *An. campestris*-like (Chiang Mai strain), *An. barbirostris* species A1, A2, A3, and A4 of Thai populations were discovered (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009) and tested for susceptibility to indigenous strains of *P. vivax* (Thongsahuan et al. 2011). The results showed that *An. campestris*-like (Form B and E, Chiang Mai strain) was a high potential vector whereas *An. barbirostris* species A1, A2, and A3 were low potential vectors. For *An. barbirostris* species A4, it was a refractory vector for *P. vivax*. As all are morphologically indistinguishable, incorrect identification of individual members in the complex may result in failure to distinguish between a vector and non-vector species, and lead to the complication and/or unsuccessful formation of vector control strategies. Previous studies on the comparison of ITS2, COI and COII nucleotide sequences of the five sibling species members of *An. barbirostris* complex revealed that only ITS2 sequences showed large sequence divergences. Although the ITS2 sequence of species A4 (1,676 bp) can be used to distinguish species A1 (1,861 bp), A2 (1,717 bp), and A3 (1,070 bp), it is slightly different from *An. campestris*-like (1,651 bp) (Saeung et al. 2007, 2008; Suwannamit et al. 2009). Thus, it is difficult to differentiate the size of the ITS2-PCR products between species A4 and *An. campestris*-like on 0.8% agarose gel.

In this study, we collected mosquitoes in the same and other localities for the *An. barbirostris* complex in eight provinces in Thailand to confirm the distribution of each species. The result correlates with previous collection data (Saeung et al. 2007, 2008; Suwannamit et al. 2009; Thongsahuan et al. 2009). *An. barbirostris* species A1 and A2 were widely distributed in

sympatry in low land areas in the north, northeast, south, and central Thailand, whereas species A3 and A4 were confined to Kanchanaburi and Chiang Mai, respectively. *An. campestris*-like was found at a lower altitude in rice paddy fields in San Sai District, Chiang Mai. Species A4 was detected in sympatry with species A1 at high altitude near forested foot hills of Maetang District, Chiang Mai, about 30 km from San Sai District.

In blood-sucking insects, electrophoretic profiles of salivary proteins were able to distinguish phlebotomine sandfly species (Volf et al. 2000). Also, electrophoresis of salivary heme proteins could be used to identify morphologically similar *Rhodnius* species (Soares et al. 1998, 2000). For mosquitoes, only electrophoretic protein profiles of female salivary glands of *An. darlingi* from three different geographical regions of Brazil [Dourado, Sao Paulo State (22°06′S, 48°19′W), Peixoto de Azevedo, Mato Grosso State (10°06′S, 55°31′W), Porto Velho, State of Rondonia (8°49′S, 63°54′W)] were compared (Moreira et al. 2001). Some differences of the protein profiles were observed. The authors used the electrophoretic profiles to support the evidence from cytological studies, biting cycle, and sequence analysis of ITS2, and suggested that the Dourado *An. darlingi* population may be a morphologically similar sibling species related to the Peixoto de Azevedo and Porto Velho groups (Moreira et al. 2001).

In this study, the salivary glands of female mosquitoes of the five sibling species in the *An. barbirostris* complex were analyzed by SDS-PAGE. The electrophoretic protein profiles of the salivary gland proteins differed among species. The variability in major proteins among species was observed in three molecular mass ranges. In each sibling species, the lowest major protein band (marker band) with molecular mass in 10-13 kDa range showed remarkably different relative mobility on SDS-polyacrylamide gels. But in *An. darlingi*, a different profile was only detected in 50-58 kDa range and protein with molecular mass of about 55 kDa showed different relative mobility in each sibling species (Moreira et al. 2001). As the salivary gland protein profiles of *An. darlingi* were performed in 10% SDS-PAGE, the proteins with molecular masses less than 18 kDa cannot be compared. Differences in the major proteins in the female salivary glands of *An. barbirostris* complex and *An. darlingi* indicate that each sibling species has at least one major salivary gland-specific protein. Therefore, the electrophoretic protein profiles and the specific protein band might be useful for construction of an additional tool to distinguish the five sibling species.

NanoLC-MS analysis of the marker band in each sibling species of *An. barbirostris* complex revealed that the protein of *An. barbirostris* species A1 and *An. campestris*-like matched a gSG6 protein of *An. gambiae* and of species A3 matched a gSG6 salivary protein of *An. freeborni* but for species A2 and A4 no match was found with any *Anopheles* sequences after two repeats. This result does not correlate with an observation that gSG6 is conserved in five species members of the *An. gambiae* complex, i.e., *An. gambiae*, *Anopheles melas*, *Anopheles bwambae*, *Anopheles quadriannulatus A*, and *Anopheles arabiensis* (Lombardo et al. 2009). The gSG6 protein was originally identified in *An. gambiae* in the form of a transcript specifically expressed in adult female salivary glands and predicted to encode a small secretory protein (Lanfrancotti et al. 2002). The corresponding 10 kDa protein was highly expressed in the salivary glands of adult females (Francischetti et al. 2002). Lombardo et al (2009) demonstrated that gSG6 was expressed in distal-lateral lobes and secreted with the saliva while the female mosquito probes for feeding. Injection of *gSG6* dsRNA into adult *An. gambiae* females resulted in decreased gSG6 protein levels, increased probing time and reduced blood feeding ability. The authors concluded that gSG6 plays some essential blood feeding role in female mosquitoes. gSG6 orthologs have been found so far in *An. freeborni* (subgenus *Anopheles*), the five species members of *An. gambiae* complex, *An. stephensi*, and *Anopheles funestus* (subgenus *Cellia*) (Lombardo et al. 2009). But gSG6 orthologs have not been retrieved in the transcriptomes of the Culicinae subfamily members, i.e., *Culex pipiens quinqefasciatus, Aedes aegypti*, and *Aedes albopictus* (Calvo et al. 2007; Ribeiro et al. 2004, 2007). These observations suggested that most probably gSG6 is a salivary protein specific to members of the Anophelinae subfamily. Recently, analysis of the salivary transcriptome of *An. darlingi*, a member of the *Nyssorhynchus* subgenus, revealed that gSG6 is absent in the data set (Calvo et al. 2004, 2009). In the case of *An. barbirostris* complex (subgenus *Anopheles*), therefore, further insights into the sialotransciptomes of the five sibling species may allow confirmation of the expression of gSG6 gene in the *An. barbirostris* complex and help bring a deeper understanding of the evolutionary history of the gSG6 gene.

Polymorphism of the protein profiles mosquito within species was only noted at 36 kDa protein of *An. barbirostris* species A4. This suggests that salivary proteins of this species might be more complex than those of other sibling species. Transcriptome and proteome analysis of this

species and investigation of the degree of intra-specific salivary gene polymorphism in natural mosquito populations should be performed to clarify this issue.

The distribution of specific proteins in the different morphological regions of female salivary glands has been described in various mosquitoes, for example, *An. gambiae* (Brenan et al. 2000; Lombardo et al. 2009), *An. stephensi* (Suwan et al. 2002), and *An. cracens* (formerly *An. dirus* B, Jariyapan et al. 2007). The protein profiles of the *An. barbirostris* complex in different salivary gland lobes showed similar distribution patterns to other *Anopheles* mosquitoes. Previous works with *Ae. aegypti*, *Ae. albopictus*, and *An. gambiae* indicated that the distal region of the lateral lobes synthesize and accumulate molecules that help in blood feeding. Examples are apyrase of *Ae. aegypti* and *Ae. albopictus* and gSG6 of *An. gambiae* (Rossignol et al. 1984; Marinotti et al. 1996; Lombardo et al. 2009). Although the salivary compositional diversity of several mosquito species is rapidly being revealed with the development of transcriptome analysis, the majority of these proteins have no known function; particularly the proteins expressed specifically in the median lobe. Identification of the proteins expressed specifically in each salivary gland lobe of the *An. barbirostris* complex by two-dimensional gel electrophoresis and mass spectrometry is in progress in our laboratory.

In conclusion, we report here for the first time on electrophoretic protein profiles of female salivary glands of the five sibling species in the *An. barbirostris* complex. The protein profiles differed among the sibling species. These results might be useful for construction of an additional tool to distinguish the five sibling species and lead to further study on the evolution of blood feeding and pathogen transmission. Because of differences in their roles in the transmission of malaria and filariasis (Iyengar 1953), description of the salivary proteomes and transcriptomes of the *An. barbirostris* complex is required. Comparative analysis of the proteomes/transcriptomes of the sibling species may supply better tools for determination of phylogeny of closely related species, population structure and speciation processes, and ultimately, identification of genes related to vectorial capacity and host preference.

PAPER 2: Proteomic analysis of salivary glands of female *Anopheles barbirostris* species A2 (Diptera: Culicidae) by two-dimensional gel electrophoresis and mass spectrometry

RESULTS

Age dependence of salivary gland protein profiles

Figure 1 shows the 2-DE gels of salivary gland samples collected at 0-12, 24-36, and 48-60 hours after emergence. Two-dimensional gel electrophoresis protein profile of the female salivary glands at 0-12 hours after emergence revealed 18 protein spots (spot number (SN) 1, 2, 4, 8-12, 14-16, 18-21, 24, 29, and 30). The proteins detected in 0-12 hours post emergence were also found in 24-36, and 48-60 hours post emergence. Spot number 3, 5-7, 23, 27 and SN17, 22, 26, 28 were first detected in the salivary glands of female mosquitoes aged 24-36 and 48-60 hour after emergence, respectively. As all major proteins were present from 48 hours after emergence, the 2-DE profile of the female salivary glands at 48-60 hours after emergence was used as a reference gel (Fig. 1c). The gel image analysis software detected approximately 75 well-resolved spots on the reference gel. Most of the proteins have a molecular weight range from 14 to 85 kDa and pI range from 3.9 to 10. Thirty protein spots were excised and subjected to nanoLC-MS for identification. The proteins were identified according to the peptide fragment digested by trypsin and matched with the theoretical standard spectra of the protein in public database (Table 1). Spot numbers in Table 1 correspond to the salivary gland protein shown in Figure 1c. Only 17 protein spots were significantly matched with protein sequences in the database. Five of the spots (SN4, 19, 21, 24 and 29) were proteins involving blood-feeding function. The expression volume of the salivary gland proteins after emergence of *An. barbirostris* species A2 is shown in Table 2. Expression volumes of 20 spots increased and two spots decreased within 60 hours of mosquito development. From the 20 spots, ten spots increased in volume more than 1000 fold. Among them, SN20, long form D7 protein (SN21), and D7-related 1 protein (SN24) had a high expression rate, respectively. The spots decreased in volume were proteins with unknown function, SN8 and 9.

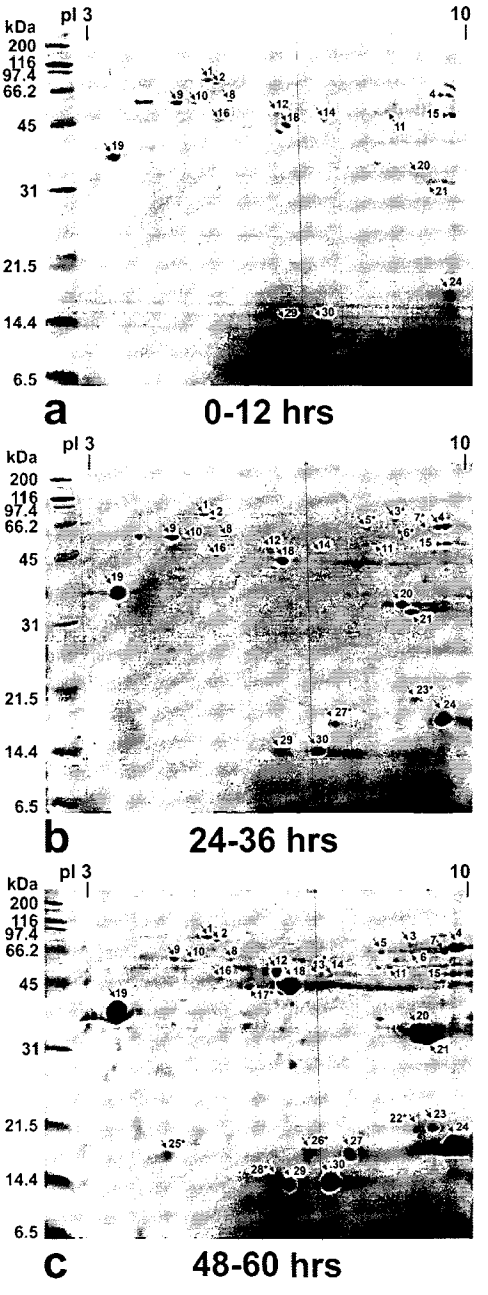

Fig. 1 Two-dimensional gel analysis of female salivary gland proteins of *An. barbirostris* species A2 mosquitoes according to age. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie blue. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. Numbers indicate major salivary gland proteins. **a:** a representative of 2-D gels of proteins extracted from 70 female mosquitoes aged 0-12 hours; **b:** 24-36 hours; **c:** 48-60 hours.

Table 1 NanoLC-MS identification of salivary gland proteins from female *An. barbirostris* species A2

SN[a]	Accession number[b]	Protein description [Species]	Database MW/pI	Protein score[d]	No. of peptides/% coverage	First detection by 2-DE (hour)[e]	Salivary gland region[f]	Classification
1	gi|94468818	Heat shock cognate 70 [*Aedes aegypti*]	72.356/ 5.06	753	12/20	0	P, D, M	Housekeeping
2	gi|307176326	Heat shock 70 kDa protein cognate 4 [*Camponotus floridanus*]	71.666/ 5.43	318	7/12	0	P, D, M	Housekeeping
3	NSH[c]					24	P	
4	gi|208657633	Putative 5' nucleotidase/apyrase [*An. darlingi*]	63.527/ 8.72	114	3/4	0	D, M	Blood feeding
5	gi|157133637	Bifunctional purine biosynthesis protein [*Ae. aegypti*]	64.640/ 8.21	128	4/6	24	D, M	Housekeeping
6	NSH					24	D, M	
7	NSH					24	D, M	
8	gi|118778070	AGAP007393-PB [*An. gambiae* str. PEST]	54.791/ 5.58	195	5/10	0	P, D, M	Unknown
9	gi|158300147	AGAP012407-PA [*An. gambiae* str. PEST]	53.384/ 5.02	483	11/22	0	P, D, M	Unknown
10	gi|94468834	F0/F1-type ATP synthase beta subunit [*Ae. aegypti*]	53.937/ 5.03	776	13/34	0	P, D, M	Housekeeping

11	gi|170032139	Conserved hypothetical protein [*Culex quinquefasciatus*]	59.442/ 9.01	443	7/15	0	P, D, M	Unknown
12	NSH					0	D^g, M	
13	gi|58386650	AGAP008802-PA [*An. gambiae* str. PEST]	44.042/ 7.01	236	6/15	48	D, M	Unknown
14	gi|58390364	AGAP007827-PA [*An. gambiae* str. PEST]	46.883/ 6.43	85	3/10	0	D, M	Unknown
15	NSH					0	M	
16	gi|207298829	Skeletal muscle actin 3 [*Homarus americanus*]	42.177/ 5.17	60	2/7	0	P, M	Housekeeping
17	NSH					48	D^g, M	
18	gi|170041072	Conserved hypothetical protein [*C. quinquefasciatus*]	48.251/5.85	32	1/2	0	D^g, M	Unknown
19	gi|190576759	Anti-platelet protein [*An. gambiae*]	27.172/ 4.13	92	1/4	0	P^g, D	Blood feeding
20	NSH					0	P^g, D, M	
21	gi|114864717	Long form D7 salivary protein [*Anopheles funestus*]	36.720/ 8.45	47	2/5	0	P^g, D, M	Blood feeding
22	NSH					48	D	
23	gi|158289973	AGAP010375-PA [*An. gambiae* str. PEST]	17.651/ 10.43	73	3/11	24	D	Unknown
24	gi|4538887	D7-related 1 protein [*An. gambiae*]	19.053/ 9.24	59	2/9	0	D	Blood feeding
25	NSH					48	D	

26	NSH					48	D	
27	NSH					24	D	
28	NSH					48	D, M	
29	gi|229418592	gSG6 salivary protein [*Anopheles freeborni*]	13.940/ 6.28	41	1/5	0	D	Blood feeding
30	NSH					0	M	

[a]Spot number refers to those shown in Fig. 1

[b]Accession number of the hit of proteins from mosquitoes or other arthropod species

[c]NSH: Not significant hit

[d]$P < 0.05$ or MASCOT score ≥ 30

[e]Time post emergence that the protein was firstly detected by 2D electrophoresis.

[f]Protein expressed in different portions of the female salivary glands: P, Proximal-lateral lobe; D, Distal-lateral lobe; M, Median lobe

[g]Low amount of expression

Table 2 List of expression volume of 30 protein spots in the female salivary gland of *An. barbirostris* species A2 at different time post emergence

SN[a]	MW[b]	pI[c]	0-12 hrs[d]		24-36 hrs[e]		48-60 hrs[f]	
			ANV[g]	Index[h]	ANV	Index	ANV	Index
1	85	5.30	0.23	100.00	0.23	100.00	0.23	100.00
2	75	5.46	0.05	100.00	0.05	100.00	0.05	100.00
3	69	9.29	-	-	0.03	100.00	0.06	200.00
4	66	10.00	0.07	100.00	0.55	785.71	1.74	2485.71
5	64	8.81	-	-	0.02	100.00	0.14	700.00
6	64	9.31	-	-	0.01	100.00	0.07	700.00
7	64	9.85	-	-	0.02	100.00	0.31	1550.00
8	59	5.72	0.04	100.00	0.03	75.00	0.03	75.00
9	58	4.79	0.31	100.00	0.31	100.00	0.24	77.42
10	58	5.05	0.03	100.00	0.03	100.00	0.05	166.67
11	55	8.95	0.01	100.00	0.03	300.00	0.06	600.00
12	53	6.50	0.02	100.00	0.03	150.00	0.86	2866.67
13	51	7.69	-	-	-	-	0.05	100.00
14	51	7.97	0.02	100.00	0.02	100.00	0.07	350.00
15	51	10.00	0.19	100.00	0.20	105.21	0.26	136.84
16	49	5.53	0.02	100.00	0.02	100.00	0.08	400.00
17	45	6.04	-	-	-	-	0.35	100.00
18	45	7.00	0.23	100.00	0.63	273.91	4.56	1982.61
19	35	3.87	0.38	100.00	2.24	589.47	6.16	1621.05
20	33	9.39	0.03	100.00	0.49	1633.33	3.98	13266.67
21	32	9.64	0.04	100.00	0.39	975.00	3.57	8925.00
22	20	9.42	-	-	-	-	0.32	100.00
23	20	9.68	-	-	0.05	100.00	0.61	1220.00
24	18	10.00	0.20	100.00	1.36	680.00	8.53	4265.00
25	17	4.70	-	-	-	-	0.12	100.00
26	17	7.50	-	-	-	-	0.38	100.00
27	17	8.31	-	-	0.14	100.00	1.11	792.86
28	14	6.49	-	-	-	-	0.41	100.00
29	14	7.00	0.07	100.00	0.17	242.86	1.31	1871.43
30	14	7.99	0.20	100.00	0.60	300.00	4.15	2075.00

[a]Spot number refers to those shown in Fig. 1

[b]MW: observed molecular mass

[c]pI: observed isoeletric point

[d]0-12 hours post emergence

[e]24-36 hours post emergence

[f]48-60 hours post emergence

[g]ANV: average normalization volume of protein spot

[h]Index: relative volume expressed on the hour when the relatively average normalization volume of protein was expressed at the first hour

Protein profiles of different regions of the female salivary glands

The results of the 2-DE gels of total proteins in the proximal-lateral lobes, distal-lateral lobes and median lobes of the female salivary glands of *An. barbirostris* species A2 are shown in Figure 2. The different morphological regions of the female salivary glands displayed distinct electrophoretic protein profiles. Eleven protein spots (SN1-3, 8-11, 16, and 19-21) were found in the proximal-lateral lobes. Twenty-six protein spots (SN1, 2, 4-14, and 17-29) appeared in the distal region of the lateral lobes, while twenty-one protein spots (SN1, 2, 4-18, 19, 20, 28, and 30) were detected in the medial lobe. Spot number 3 was only a specific protein in this lobe but it was not significantly matched with protein sequences in the database. Spot number 22-27 and SN29 were expressed only in the distal-lateral lobes. In the medial lobe, SN15 and SN30 were expressed specifically in this lobe. Eight (SN1-2, 8-11, and 20-21), 10 (SN4-7, 12-14, 17-18, and 28), and one (SN16) protein spots were expressed in all lobes, distal-lateral and medial lobes, and proximal-lateral and medial lobes, respectively (Fig. 2 and Table 1).

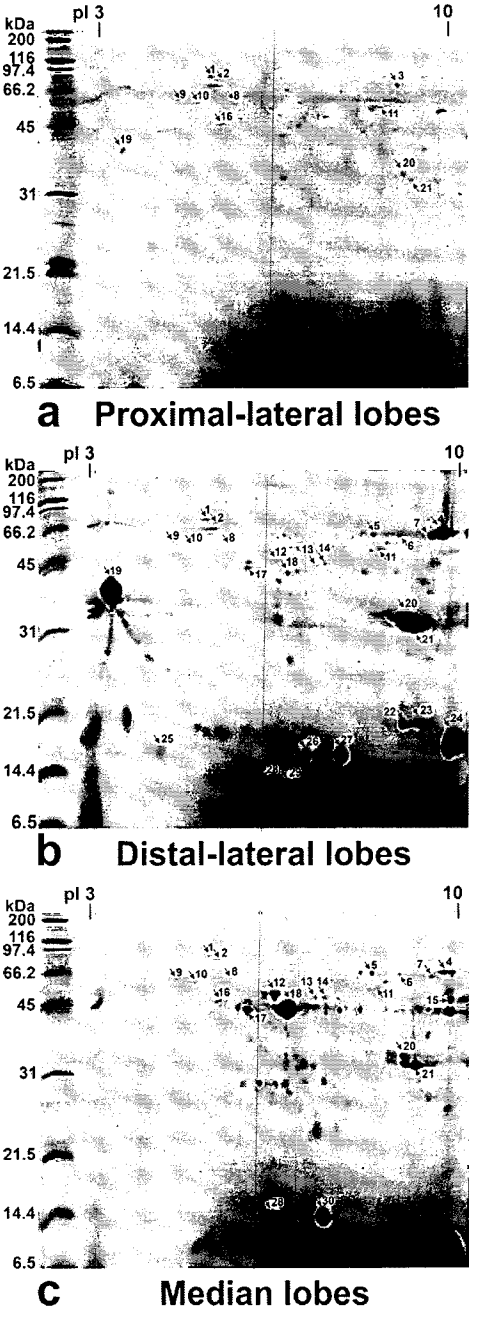

Fig. 2 Two-dimensional gel analysis of proteins expressed in the different regions of *An. barbirostris* species A2 female salivary glands. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie blue. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. Numbers indicate major salivary gland proteins. **a:** a representative of 2-D gels of proteins extracted from proximal-lateral lobes of 70 female mosquitoes aged 48-60 hours; **b:** distal-lateral lobes; **c:** median lobe.

DISCUSSION

The saliva of mosquitoes contains different sets of enzymes for various functions, for example, feeding, immune modulation, defending pathogens, and metabolic functions. The physiological state of the mosquitoes has been shown to be an important factor in salivary protein amount and composition (Poehling 1979; Nascimento et al. 2000). Our study showed that the proteome profiles of *An. barbirostris* species A2 female salivary glands were affected by ageing. The expression of most of salivary gland proteins in females varied from zero to 60 hours post emergence. Some proteins involved in blood-feeding, housekeeping, or unknown functions.

For proteins involved in blood feeding, i.e., putative 5' nucleotidase/apyrase, anti-platelet protein, long form D7 salivary protein, D7-related 1 protein, and gSG6 salivary protein started to accumulate after emergence and gradually increased from hour zero and become predominant proteins within 48 hours. The results suggested that the salivary gland proteins of female *An. barbirostris* species A2 reached the mature stage for feeding on blood within 48 hours. Our results are consistent with previous studies in *An. darlingi*, *An. stephensi*, *Culex pipiens* and *C. quinquefasciatus* (Moreira et al. 2001; Poehling 1979; Nascimento et al. 2000). Moreira et al (2001) reported the SDS-PAGE pattern of the salivary proteins from sugar-fed female *An. darlingi* mosquitoes with ages varying from one to ten days after adult emergence. The protein pattern of female *An. darlingi* salivary glands does not vary qualitatively during the first ten days of adult life. The differences in the amount of proteins in the profiles of each age reflect an increase in the amount of protein in the glands of older mosquitoes. In *An. stephensi*, *C. pipiens* and *C. quinquefasciatus*, the major polypeptides are present in the salivary glands since the first day of adult life (Poehling 1979; Nascimento et al. 2000). In *Culex molestus* and *Ae. aegypti*, the protein profile of the salivary glands of recently emerged females reveals few polypeptides and only in the third day after emergence can all the major polypeptides be detected (Racioppi and Spielman 1987; Al-Ahdal et al. 1990).

Apyrases are nucleoside triphosphate-diphosphohydrolases present in a variety of organisms. In *Ae. aegypti*, the salivary apyrase was identified as a member of the 5' nucleotidase family (Champagne et al. 1995). Recently, apyrase of *Ae. albopictus* was cloned and characterized (Dong et al. 2012). It is an enzyme that helps the acquisition of blood meals by the degradation of adenosine diphosphate (ADP), a mediator of platelet aggregation and

inflammation (Ribeiro and Francischetti 2003) and prevents neutrophil activation (Sun et al. 2006). Smartt et al (1995) showed that apyrase protein levels peak in the salivary glands about four days after adult emergence and remain high after a blood meal. The biochemical analyses of the salivary glands of female *An. darlingi* (Marinotti et al. 1996) and *An. dirus* B (Jariyapan et al. 2007) revealed the presence of apyrase activity in the distal region of the lateral lobes (~66%), median lobe (~23%) and proximal region of the lateral lobes (~11%). In this study, *An. barbirostris* species A2 apyrase was found only in distal-lateral and medial lobes. No apyrase was detected in the proximal-lateral lobe by 2-DE approach. Characterization of apyrase activity in the *An. barbirostris* species A2 would help to clarify this issue.

One of the most abundant families of mRNAs expressed in the salivary glands of mosquitoes includes those encoding products related to the 30-kDa allergen of *Ae. aegypti* (Simons and Peng 2001). The cDNA sequence of the gene was first reported by Xu et al (1998), and conceptual translation revealed two distinct domains, an acidic, low complexity domain rich in glycine (G), glutamic acid (E), and aspartic acid (D) residues and a more complex carboxyterminal domain; thus, in anopheline mosquitoes these proteins have also been called GE-rich proteins (Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cázares-Raga et al. 2007). Recently, a unique anti-platelet protein, anopheline anti-platelet protein (AAPP), from the salivary gland of female *An. stephensi* was identified by Yoshida et al (2008). AAPP specifically blocks platelet adhesion to collagen by binding directly to collagen and subsequently aggregating platelets. Hayashi et al (2012) investigated the *in vivo* anti-thrombotic effects of AAPP and suggested that AAPP has great potential as a new anti-platelet agent with a better risk/benefit ratio than that seen with aspirin. The members of the GE-rich/anti-platelet protein/30-kDa allergen family in mosquitoes have apparent molecular masses ranging from 30 to 35 kDa when separated on (SDS)-polyacrylamide gels. The richness of acidic residues confers a pI for this protein family in the range of 3.9-4.6 (Ribeiro et al. 2010). In *An. barbirostris* species A2, an anti-platelet protein was found as one of the most abundantly expressed acidic proteins in the female salivary glands as in all anopheline mosquitoes studied so far. Although it was detected predominantly in the distal-lateral lobes of the salivary glands, the small amount was also detected in the proximal-lateral lobes. This might be due to the accumulation of the protein in the

lumen of the salivary duct as this protein might act as a lubricant of food during both sugar and/or blood feeding as proposed by Jariyapan et al (2006).

Proteins of the D7 family are distantly related to the OBP super-family and present in the saliva or salivary glands of numerous female blood-sucking insects (James et al. 1991; Arca et al. 1999a; Valenzuela et al. 2002; Bahia et al. 2007; Geng et al. 2009). D7 proteins are one of the abundant proteins in the saliva of female mosquitoes and have been proposed to inhibit hemostasis by trapping agonists of hemostasis (Calvo et al. 2006a). The D7 protein exists in two forms: a long form (~30-35 kDa), which is found exclusively in mosquitoes and sand-flies, and the short forms (~15 kDa), which are found in other insects (Valenzuela et al. 2002; Arca et al. 2002). In *An. gambiae* female salivary glands, five different D7-related (D7r1, 2, 3, 4, and 5) short forms and three D7 long forms have been identified (Arca et al. 2002; Arca et al. 2005). The D7r1, 2, 3, 4 and D7 long forms have been shown to bind to the biogenic amines serotonin, histamine, and norepinephrine (Arca et al. 2005; Calvo et al. 2006). One short D7 protein from *An. stephensi*, hamadarin (D7r1), has been shown to inhibit the plasma contact system by preventing the activation of kallikrein by Factor XIIa (Isawa et al. 2007). Das et al (2010) used RNAi-mediated gene silencing method to assess the role of *D7L2* gene product in the blood-feeding process in *An. gambiae*. Results showed that the blood-feeding capacity was low after the silencing of *D7L2* that strongly supports the involvement of D7L2 and other members of D7 protein family in the blood-feeding process. In our study, 2-DE followed by LC-MS identified only one D7 long form and one D7 short form in *An. barbirostris* species A2. However, the 2D maps showed two more spots near the D7 long form and two more spots near the D7 short form. The spots might be the other D7 long and shot forms. Further identification with other proteome and/or transcriptome approaches may elucidate this issue. The D7 long form found predominantly in the distal-lateral and medial lobes of the *An. barbirostris* species A2 salivary glands correlates with a previous study in *An. stephensi* (Suwan et al. 2002). For the D7 short form, it was expressed only in the distal-lateral lobes as detected in *Ae. aegypti* (Juhn et al. 2011).

Another protein involved in blood feeding is gSG6. It was detected in the distal-lateral lobes of the salivary gland of *An. barbirostris* species A2. This result correlates with a study in *An. gambiae* that gSG6 was expressed only in distal-lateral lobes (Lombardo et al. 2009). The gSG6 protein was first identified in the female *An. gambiae* mosquito (Lanfrancotti et al. 2002). It

was conserved in five species members of the *An. gambiae* complex, i.e., *An. gambiae*, *Anopheles melas*, *Anopheles bwambae*, *Anopheles quadriannulatus* A, and *Anopheles arabiensis* (Lombardo et al. 2009). The protein was secreted with the saliva while the female mosquito probes for feeding. Injection of *gSG6* dsRNA into adult *An. gambiae* females resulted in decreased gSG6 protein levels, increased probing time and reduced blood feeding ability (Lombardo et al. 2009). Lombardo et al. (2009) concluded that gSG6 plays some essential blood feeding role in female mosquitoes. Recently, the gSG6-P1 peptide from gSG6 protein of *An. gambiae* salivary glands was designed as a specific salivary sequence of malaria vector species. It was shown that the quantification of human antibody (Ab) responses to *Anopheles* salivary proteins in general and especially to the gSG6-P1 peptide was a pertinent biomarker of human exposure to *Anopheles* (Poinsignon et al. 2008; Drame et al. 2010).

Two proteins, F0/F1-type ATP synthase and skeletal muscle actin 3, involved in housekeeping functions were identified in *An. barbirostris* species A2. They were found from zero hours after emergence and their amount increased gradually within 48 hours. F0/F1-type ATP synthase was detected in 2-DE maps of female *Ae. aegypti* salivary glands (Ribeiro et al. 2007) and in 1-D gel of *An. gambiae* female salivary glands (Kalume et al. 2005a). It is capable of catalyzing ATP hydrolysis. Since ATP may synergize with ADP as a platelet-aggregating agent, ATP hydrolysis may play a role in blood-feeding mechanisms (Packham and Mustard 2005). Ribeiro et al (2007) proposed that the protein could be involved in energy metabolism associated with protein synthesis and secretion promoting the blood-feeding or could act as inhibitors of immune-response because ATP is known to be a signal for neutrophil activation. Skeletal muscle actin 3 is a constituent of cytoskeleton and muscle fibers. Actin is also one of three classes of the cytoskeleton, i.e., microfilament, intermediate filament, and microtubule, found in the cytoplasm of all cells. It plays important roles in cellular motion, intracellular transport, and cell division and differentiation (Khaitlina 2001). Differential actin expression in salivary glands of sugar-feeding and blood-feeding *Ae. aegypti* mosquitoes was studied by immunofluorescence (Wasinpiyamongkol et al. 2010). Results demonstrated that actin seemed to localize to the cell boundary and along the duct of each lobe of salivary glands in both sugar-feeding and blood-feeding groups. No difference was observed between the two groups in

morphology, condition, or cytoskeletal organization. Wasinpiyamongkol et al (2010) concluded that after blood meal there is no disruption of the salivary gland cytoskeleton of *Ae. aegypti.*

Two spots that matched heat shock 70 kDa proteins (Hsp70s) were found from the beginning of adult life and their amounts remained unchanged in 60 hours post emergence. The Hsp70s are a family of ubiquitously expressed heat shock proteins. They are an important part of the cell's machinery for protein folding, and help to protect cells from thermal or oxidative stress. These stresses normally act to damage proteins, causing partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues exposed by stress, Hsp70 prevents these partially-denatured proteins from aggregating, and allows them to refold. Niedzwiecki et al (1991) demonstrated that expression of Hsp70s in *Drosophila melanogaster* is regulated by the accumulation of conformationally altered proteins in old insects. The level of Hsp70 mRNA increased in files up to 23-28 days of age, but then declines as the insects get older. Hsp70s are also found in the salivary glands of *Ae. aegypti* (Thangamani and Wikel 2009) and *An. gambiae* (Kalume et al. 2005a; Wang et al. 2010).

One spot matched a bifunctional purine biosynthesis protein of *Ae. aegypti* was detected from 24 hour post emergence. However, the function of the protein in mosquitoes has not been reported. In human, bifunctional purine biosynthesis protein PURH is a protein that is encoded by the *ATIC* gene (Rayl et al. 1996). ATIC is a bifunctional enzyme involved in the purine biosynthesis pathway. One of the activities of ATIC is AICAR transformylase (AICAR Tfase), which catalyzes the formylation of 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) by N-10-formyl-tetrahydrofolate (10-formyl-THF) to produce formyl-AICAR (FAICAR) and THF. ATIC also acts as an IMP (inosine monophosphate) cyclohydrolase (IMPCH), which converts FAICAR to IMP in the final step of *de novo* purine biosynthesis (Boccalatte et al. 2009).

Five and two protein spots significantly matched with sequences of hypothetical proteins of *An. gambiae* and *C. quinquefasciatus*, respectively. Two of them, AGAP007393-PB [*An. gambiae* str. PEST] (SN8) and AGAP012407-PA [*An. gambiae* str. PEST] (SN9), decreased in volume after emergence. However, the function of the proteins is still unknown as their sequences have no obvious protein domain or motif that can provide some clues regarding their function. Thirteen protein spots were not significantly matched with protein sequences in the database. These proteins should be identified with other proteomic approaches, for examples, 1-DE

followed by LC MS/MS, in-solution followed by LC MS/MS, and iTRAQ labelling. These approaches may help to identify more proteins in the salivary glands as the previous studies in *An. gambiae* (Kalume et al. 2005a; Choumet et al. 2007).

Recently, Junh et al (2011) reported the hybridization *in situ* patterns of 30 genes expressed in the salivary glands of adult female *Ae. aegypti*. Salivary gland genes expressed in the proximal-lateral lobes and involving in sugar-feeding and bacteriocidal function are *alpha-glucosidase*, *amylase 1*, *lysozyme*, and *gambicin*. Genes involving with blood feeding, *D7 short 2*, *30 kDa*, and an *antigen-5 family member*, are expressed only in the distal-lateral lobes while, *salivary apyrase*, *D7 long 1*, *D7 long 2*, and *salivary purine nucleotidase* are expressed both in the distal-lateral and medial lobes (Juhn et al. 2011). In *An. barbirostris* species A2, proteins involved in blood feeding were also detected in the distal-lateral lobes and/or medial lobes as discussed above. These results confirm the role of distal-lateral and medial lobes in blood feeding. In this study, SN22, 25-26, and 28 proteins expressed only in the distal-lateral lobes and started accumulate from 48 hours post emergence were not significantly matched with protein sequences in the database. It is interesting to identify and characterize them in the future. These proteins may have a role in blood feeding and/or involved in pathogen transmission.

In conclusion, the proteins in the salivary glands of female *An. barbirostris* species A2 were analyzed for the first time using a proteomic approach (2-DE followed by nanoLC-MS). The proteome profiles of *An. barbirostris* species A2 female salivary glands were affected by ageing. As feeding on blood of mosquitoes depends on maturation of salivary glands and saliva contents, the salivary glands of female *An. barbirostris* species A2 are mature from 48 hours post emergence. Proteins involved in blood feeding started to accumulate from zero hours after emergence and gradually increased and became predominant within 48 hours. The different morphological regions of the female salivary glands (proximal-lateral lobes, distal-lateral lobes and median lobes) displayed distinct electrophoretic protein profiles. Proteins detected and/or identified by this approach could be tested in strategies developed to control pathogen and disease transmission. In addition, this study provides a 2D map of the *An. barbirostris* species A2 female salivary gland that could be used to compare with other related species in the *An. barbirostris* complex (Jariyapan et al. 2010) for identification of different proteins that could be used to distinguish mosquito species members in the complex.

PAPER 3: Salivary gland proteome of the human malaria vector, *Anopheles campestris*-like (Diptera: Culicidae)

RESULTS

Amount of salivary gland proteins of male and female *An. campestris*-like mosquitoes

The total amount of salivary gland proteins in male mosquitoes aged between 3-5 days was approximately 0.1 ± 0.05 μg/male and females was 1.38 ± 0.01 μg/female (n = 25).

Proteins differentially expressed in the salivary glands of males and each salivary gland lobes of female mosquitoes and glycoprotein analysis

Twelve major protein bands of the *An. campestris*-like female salivary glands are shown in Figure 1. The male protein profile differed from the whole female profile (compare lane M with lane F) but appeared similar to the proximal-lateral region profile (lane PL). The different morphological regions of the female salivary glands also displayed distinct protein profiles. Female specific protein bands 1, 5, 6, 7, 8, 9, 10 and 12 appeared in the distal region (lane DL) whereas the protein bands 1, 2, 3, 4, 6, 7 and 11 were in the medial lobe (lane ML). For more detail analysis on the expression patterns of proteins in the male and different lobes of female salivary glands 2-DE and nanoLC-MS were performed. Each sample was subjected to triplicate runs, and the results were highly reproducible. Two-dimensional gel electrophoresis experiments provided evidence of many proteins in the female mosquito salivary glands, approximately 85 well-resolved spots (Fig. 2a). The gel (Fig. 2a) was considered the standard reference gel. The molecular mass of these spots varied from 10-72 kDa, with pI ranging of 3.9-10. Twenty major protein spots were detected in the female mosquitoes and identified by nanoLC-MS. Spot numbers in Table 1 correspond to the salivary gland proteins shown in Figure 2a. From the 20 major protein spots, 15 spots are positive for glycoprotein staining (Fig. 2b and Table 1). Expression patterns of the protein spots in male and different lobes of female salivary glands are shown in Figure 3 and Table 1. Ten major protein spots (SN1, 2, 3, 4, 6, 8, 11, 12, 15, and 19) were similarly found with weak intensity in the male salivary glands (Fig. 3a) and the proximal-lateral lobes of female glands (Fig. 3b). Five major protein spots (SN13, 14, 16, 17, and 20) were

detected only in the distal-lateral lobes (Fig. 3c) whereas three major spots (SN5, 7, and 18) were specific in the medial lobe (Fig. 3d).

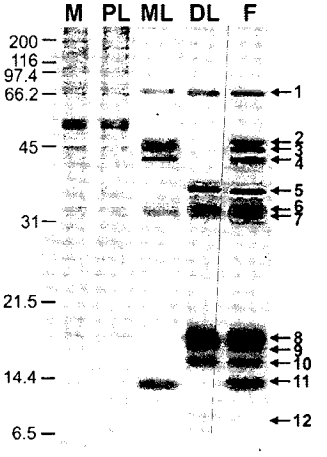

Fig. 1 Female and male salivary gland protein profiles of *An. campestris*-like. The salivary gland proteins were separated on 15% SDS-polyacrylamide gels and stained with CBB. M: sixty male salivary glands; PL: fifty female proximal-lateral lobes; ML: two median lobes; DL: two distal-lateral lobes; F: two whole female salivary glands. Molecular mass markers are indicated on the left in kDa. Arrows indicate major salivary gland proteins of female mosquitoes.

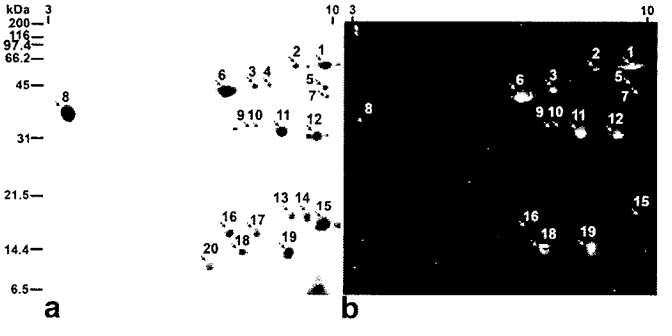

Fig. 2 Two-dimensional gel analysis of female salivary gland proteins of *An. campestris*-like. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. Numbers indicate major salivary gland proteins. **a:** representative of 2-DE gels stained with CBB; **b:** representative of 2-DE gels stained with Pro-Q Emerald 300 glycoprotein stain.

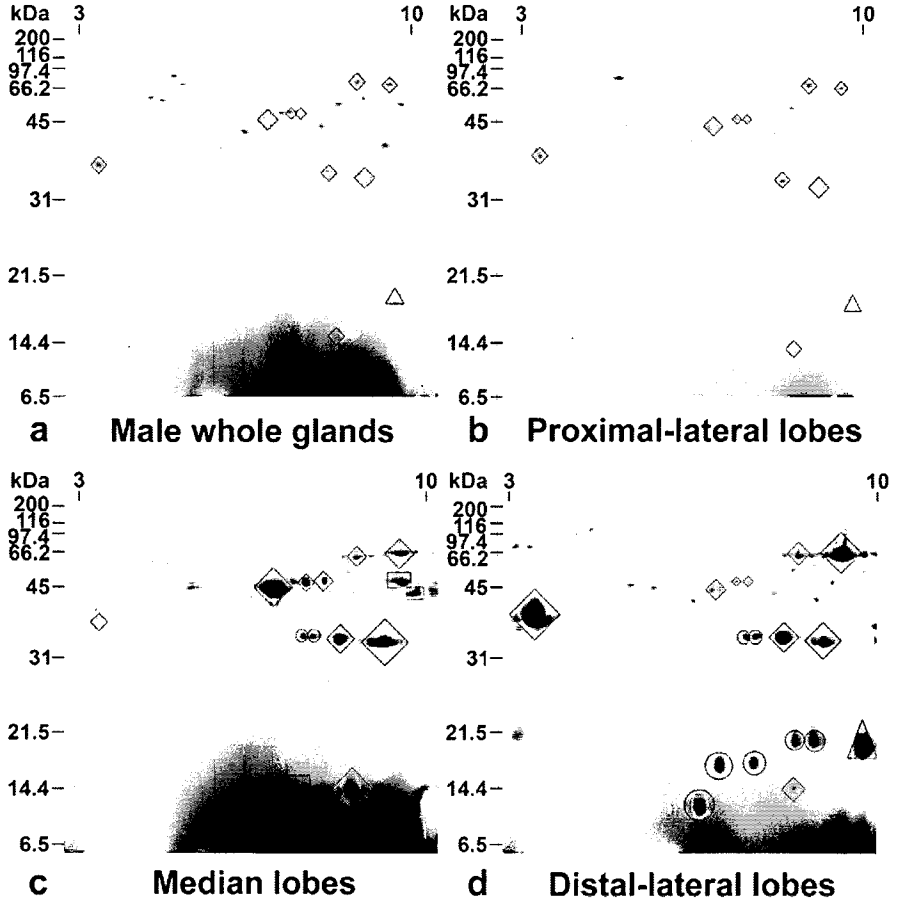

Fig. 3 Two-dimensional gel analysis of proteins expressed in male and different regions of female *An. campestris*-like salivary glands. Proteins were separated on Immobiline DryStrips 7 cm, pI 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with CBB. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. **a:** representative of 2-DE gels of proteins extracted from male glands; **b:** proximal-lateral lobes; **c:** medial lobes; **d:** distal-lateral lobes. ○ = a major spot found in distal-lateral lobe, □ = a major spot found in medial lobe, △ = a major spot found in male, proximal-lateral and distal-lateral lobes, ▢ = a major spot found in medial and distal-lateral lobes, ◇ = a major spot found in male and all female lobes.

Table 1 A list of major protein spots of *An. campestris*-like salivary glands identified by NanoLC-MS

SN[a]	Accession number[b]	Protein description [species]	Protein score[d]	No. of peptides/ % coverage	Database Mw/pI	Observed Mw/pI	Exp. Pattern[e]	Glycoprotein	Classification
1	gi|4582524	apyrase [*Anopheles gambiae*]	31	1/1	62.1/8.9	68/9.8	M[f], PL[f], ML, DL	yes	Blood feeding
2	gi|208657633	putative 5' nucleotidase/apyrase [*Anopheles darlingi*]	68	2/6	63.5/8.7	67/8.0	M[f], PL[f], ML, DL	yes	Blood feeding
3	NSH[c]					56/7.5	M[f], PL[f], ML, DL[f]	yes	
4	NSH					55/7.2	M[f], PL[f], ML, DL[f]	no	
5	gi|242021351	hypothetical protein Phum_PHUM512530 [*Pediculus humanus corporis*]	35	1/3	33.7/9.4	55/9.8	ML	yes[g]	Unknown
6	gi|170046888	serine/threonine-protein kinase rio3 [*Culex quinquefasciatus*]	33	2/5	56.9/9.4	52/6.7	M[f], PL[f], ML, DL[f]	yes	Housekeeping
7	NSH					50/9.6	ML	yes[g]	
8	gi|190576759	anti-platelet protein [*An. gambiae*]	40	1/4	27.2/4.1	38/4.0	M[f], PL[f], ML[f], DL	yes[g]	Blood feeding
9	NSH					37/7.1	DL, ML	yes[g]	

10	gi|241998444	sil1, putative [*Ixodes scapularis*]	35	1/2	36.4/5.1	37/7.3	DL, ML	yes[g]	Housekeeping
11	gi|15718081	D7 protein [*Anopheles stephensi*]	41	1/3	36.9/8.8	36/7.7	M[f], PL[f], ML, DL	yes	Blood feeding
12	gi|158285343	AGAP007618-PA [*An. gambiae* str. PEST]	37	1/2	30.9/8.4	35/9.4	M[f], PL[f], ML, DL	yes	Unknown
13	gi|4538887	D7-related 1 protein [*An. gambiae*]	55	1/6	19.1/9.2	20/7.9	DL	no	Blood feeding
14	gi|241616200	cyclophilin A, putative [*I. scapularis*]	41	1/4	22.1/9.2	20/9.0	DL	no	Housekeeping
15	gi|16225961	short form D7r1 salivary protein [*Anopheles arabiensis*]	52	1/6	19/9.2	19/9.8	M[f], PL[f], DL	yes[g]	Blood feeding
16	gi|270014872	hypothetical protein TcasGA2_TC010859 [*Tribolium castaneum*]	31	1/4	16.1/7.8	17/6.8	DL	yes[g]	Unknown
17	NSH					17/7.3	DL	no	
18	NSH					14/7.0	ML	yes	
19	gi|312381960	hypothetical protein AND_05658 [*An. darlingi*]	38	1/4	16.9/7.7	14/7.9	M[f], PL[f], ML, DL[f]	yes	Unknown
20	gi|13537666	gSG6 protein [*An. gambiae*]	87	2/10	13.7/5.3	12/6.5	DL	no	Blood feeding

[a]Spot number refers to those shown in Fig. 2a

[b]Accession number of the best hit of proteins from mosquitoes and/or arthropod species

[c]NSH = not significant hit

[d]$P < 0.05$ or MASCOT score $\geq$ 30

[e]Expression pattern in male and different regions of female salivary glands: M, male; PL, proximal-lateral lobe; DL, distal-lateral lobe; ML, medial lobe

[f]Low amount of expression

[g]Low intensity

DISCUSSION

Salivary glands of male and female *Anopheles* mosquitoes are morphologically different. Salivary glands of male mosquitoes consist of a single small lobe whereas female mosquitoes are composed of two lateral lobes with distinct proximal and distal regions and a median lobe (Moreira-Ferro et al. 1999; Jariyapan et al. 2007). Their feeding success is related to salivary proteins. Male mosquitoes feed only on sugar whereas females feed on both sugar and blood. Proximal regions of the lateral lobes produce enzymes involved in sugar feeding. Molecular molecules related to blood feeding are synthesized by the distal-lateral regions and the medial lobes (James 2003). Determination of the *An. campestris*-like salivary gland extracts revealed that the male glands contained approximately ten times less protein than the female ones. These values are consistent with the morphological differences observed between the salivary glands of males and females. Also, these morphological and protein content differences have been observed in other mosquito species and are related with the different feeding habits of males and females (Moreira-Ferro et al. 1999; Nascimento et al. 2000; Siriyasatien et al. 2005; Jariyapan et al. 2007; Phumee et al. 2011).

Previous analyses of mosquito salivary glands using SDS-PAGE in *An. stephensi* (Suwan et al. 2002), *An. carcens* (formerly *An. dirus* B) (Jariyapan et al. 2007), *Anopheles albimanus* (Cázares-Raga et al. 2007) and *Anopheles barbirostris* species A2 (Jariyapan et al. 2012) demonstrated that there are approximately 12-15 major and several minor proteins in the females. In *An. campestris*-like, at least 12 major proteins were found in the female salivary glands and each morphological region of the female glands contained different major proteins. Analysis using 2-DE revealed that the salivary gland protein profile of the male was similar to that of the female proximal portion of the lateral lobes as described for *An. albimanus* (Cázares-Raga et al. 2007), suggesting that these lobes are responsible for sugar feeding. In our study, as only major proteins were selected for identification, no protein involved in the digestion of sugar was identified. An explanation is that proteins involved in sugar feeding such as alpha-glucosidase may be produced with a very small amount in the glands and collected in the crop, a sac-like compartment for the digestion of sucrose (James et al. 1989; Marinotti and James 1990; Marinotti et al. 1996; Moreira-Ferro et al. 1999). Alpha-glucosidase activities have been detected in salivary glands of *Aedes albopictus* (Marinotti et al. 1996), *An. darlingi* (Moreira-Ferro et al. 1999), and

An. carcens (Jariyapan et al. 2007), however, no activity has been detected in the crop of *Anopheles aquasalis* but in the midgut (Souza-Neto et al. 2007). Studies in phlebotomus sandflies *Phlebotomus langeroni* (Dillon and el-Kordy 1997) *Lutzomia longipalpis* (Gontijo et al. 1998), and *Phlebotomus papatasi* (Jacobson and Schlein 2001) show that alpha-glucosidase activities have also been detected in midgut but not in the crop suggesting that sugar digestion is carried out in the midgut. Thus, salivary alpha-glucosidase may be used for assisting solubilization of sugars (Eliason 1963) and for intra cellular metabolism (Dillon and el-Kordy 1997). Characterization of alpha-glucosidase activity in the *An. campestris*-like would help to clarify this issue.

Several techniques including SDS-PAGE, 2-DE and liquid chromatography tandem mass spectrometry (LC-MS/MS) are combined and used to describe *An. gambiae* salivary gland and saliva contents (Kalume et al. 2005a; Choumet et al. 2007). The studies identified five saliva proteins and 122 more proteins from the salivary glands, including the first proteomic description for 89 of these salivary gland proteins. Proteomic analyses of the salivary glands of *An. campestris*-like lead to the discovery of proteins that promote blood feeding, i.e., putative 5' nucleotidase/apyrase, anti-platelet protein, long form D7 salivary protein, D7-related 1 protein, and gSG6. They are secreted proteins and synthesized and accumulated in the distal region of the lateral lobes and medial lobe of the glands. This result is consistent with previous studies on salivary gland proteome profiles of *An. gambiae* (Kalume et al. 2005a; Choumet et al. 2007) and *An. barbirostris* species A2, a closely related species in the *An. barbirostris* complex (Jariyapan et al. 2012) and *in situ* hybridization results of genes involved with blood feeding in *Ae. aegypti* (Juhn et al. 2011).

However, Calvo et al (2006b) compared approximately 1,000 randomly sequenced clones of an adult male salivary gland cDNA library of *An. gambiae* with a previous data set of the female salivary gland cDNAs (Arca et al. 2005). Results show that female transcribed genes codes for proteins, D7L1, D7r1, D7r2, D7r3, and D7r4 which are implicated in anticlotting and anti-bradykinin production as well as biogenic binding activities (Calvo et al. 2006a; Isawa et al. 2007), antiplatelet aggregation proteins, 5' nucleotidase (5p_nuc) and apyrase (Ribeiro and Francischetti 2003; Sun et al. 2006), antithrombin protein, cE5, homologous to *An. albimanus* anophelin (Francischetti et al. 1999; Valenzuela et al, 1999), and proteins with unknown function, SG1-like 3 long, trio, gSG1b, gSG7-2, gSG7, hyp17, 30_kDa, and hyp15 are not found in the *An.*

gambiae male salivary gland cDNA library. In contrast to *An. gambiae*, the blood-feeding proteins, apyrase, putative 5' nucleotidase/apyrase, anti-platelet protein, D7, and short form D7r1 were detected in 2-DE gels with very small amount in the male salivary glands of *An. campestris*-like. Study on differentially expressed genes in the salivary glands of female and male *An. campestris*-like using Switching Mechanism At RNA Termini Polymerase Chain Reaction (SMART-PCR) followed by Suppression Subtractive Hybridization (SSH-PCR) techniques (Ghorbel and Murphy 2011) would help to elucidate this issue. Information from the study may be helpful in finding additional peptides and proteins with a function in blood or sugar feeding in mosquitoes as studies in *Culex pipiens pallens* (Chen et al. 2007) and *Anopheles anthropophagus* (Geng et al. 2009).

In this study, at least 15 glycoproteins were detected in the *An. campestris*-like female saliyary glands. However, few proteins in the saliva of *Anopheles* mosquitoes studied so far have been described as glycoproteins, i.e., 5'-nucleotidase/apyrase, anti-platelet protein (30 kDa allergen/GE-rich), and D7 protein. Secretory proteins are often glycosylated or modified by phosphorylation as they pass through the Golgi apparatus (Alberts et al. 2002). Glycoproteins contain oligosaccharide chains covalently attached to polypeptide side-chains. These glycoproteins are involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity. Glycoproteins contain three major types of oligosaccharides (glycans): N-linked, O-linked, and glycosylphosphatidylinositol (GPI) lipid anchors. Most of the proteins in saliva are glycosylated. Carbohydrates have many hydroxyl (-OH) groups that bind to water molecules, and thus increase stability. Thus the glycoproteins of saliva tend to lubricate the food chewed, in part to allow easier swallowing of food and its passage through the esophagus. For examples, mucins, which are found extensively in the sialotranscriptomes of insects, contain many short O-linked glycans (Calvo et al. 2007; Alves-Silva et al. 2010). These glycoproteins increase the viscosity of the fluids in which they are dissolved. Therefore, they are postulated to help maintain the insect mouthparts, in addition to other possible functions (Alves-Silva et al. 2010).

Most 5' nucleotidases are typically extracellular proteins bound to the membrane by glycosylphosphatidylinositol (GPI) anchors attached to their carboxyterminal domain. However, 5' nucleotidase/apyrases in several insects including *Ae. aegypti*, *Ae. albopictus*, *Culex pipiens*

quinquefasciatus, *L. longipalpis*, and *Glossina morsitans morsitans* lack the GPI anchor attachment domain, either through mutation or truncation, thus inferring that these proteins are secreted (Champagne et al. 1995; Charlab et al, 1999; Ribeiro et al. 2004; Ribeiro et al. 2007;Alves-Silva et al. 2010; Dong et al. 2012). An acidic glycoprotein of 35 kDa (GP35 ANOAL) from female salivary glands of *An. albimanus* contains several potential post-translational modifications predicted in its amino acids sequence. Among them, two potential N-glycosylation and nine potential O-glycosylation sites have been identified (Cazares-Raga et al. 2007). Putative N-glycosylation and O-glycosylation sites occur in all identified members of the 30kDa allergen family (Xu et al. 1998; Ribeiro et al. 2003; Valenzuela et al. 2003; Calvo et al. 2004; Jariyapan et al. 2006; Cazares-Raga et al. 2007; Yoshida et al. 2008). Proteins that contain N-glycosylation sites may play important roles in the induction of allergic responses (Wal 2001; Malandain 2005). For D7 family proteins, only D7 long forms of *An. gambiae*, *Anopheles stephensi*, *Anopheles arabiensis*, *Anopheles funestus*, and *Anopheles darlingi* contain glycosylation sites (Francischetti et al. 2002; Suwan et al. 2002; Valenzuela et al. 2002; Calvo et al. 2007; Calvo et al 2009). Glycoproteins in the salivary glands of mosquitoes should be studied for their structure and specific biological functions in receptor binding, cell signaling, and immune recognition that may be involved in pathogen transmission.

Three major protein spots of *An. campestris*-like salivary glands related to housekeeping proteins from other arthropod species included serine/threonine-protein kinase rio3 [*Culex quinquefasciatus*], sil1, putative [*Ixodes scapularis*], and cyclophilin A, putative [*I. scapularis*]. SN6 Protein that matched the serine/threonine-protein kinase rio3 of *Culex quinquefasciatus* (Arensburger et al. 2010) was a protein expressed abundantly in the medial lobe of female salivary glands but found with a small amount in the both region of the lateral lobes and the male salivary glands. The RIO family of atypical serine protein kinases has been first characterized in *Saccharomyces cerevisiae* (Angermayr and Bandlow 1997). It consists of enzymes that contain a unique domainwith a characteristic kinase sequence motif and usually some additional domains. At least two RIO proteins, Rio1 and Rio2, are present in organisms varying from Archaea to humans, with a third Rio3 subfamily present only in multicellular eukaryotes (Manning et al. 2002). Human Rio3 is characterized at the DNA level only, in two splice variants, and is identified as a protein up-regulated in the core of malignant melanomas (Roesch et al. 2003). To

date, no report of the purification of mosquito kinase Rio3 is available. SN 10 matched sil1, putative [*Ixodes scapularis*]. It was predominantly expressed in both the medial and distal-lateral lobes. SIL1 in *Homo sapiens* is a resident endoplasmic reticulum (ER), N-linked glycoprotein with an N-terminal ER targeting sequence, 2 putative N-glycosylation sites, and a C-terminal ER retention signal. This protein is required for protein translocation and folding in the endoplasmic reticulum (ER). It functions as a nucleotide exchange factor for the heat-shock protein 70 (HSP70) chaperone HSPA5 (Anttonen et al. 2005). SN 14 matched cyclophilin A, putative [*I. scapularis*]. It was expressed specifically in the distal-lateral lobe. Cyclophilin A also known as peptidylprolyl isomerase A is the most abundant member of the CyP subfamily of immunophilins and has a variety of intracellular functions, including intracellular signaling, protein trafficking, and the regulation of other proteins activity. In humans, cyclophilin A has been studied as a multifunctional protein that is up regulated in a variety of inflammatory conditions, such as rheumatoid arthritis, autoimmune disease, and cancer. Besides its intracellular functions, CyPA is a secreted molecule that has a physiological and pathological role in cardiovascular diseases (Satoh et al 2010). Functional analysis of sil1 and cyclophilin A in *An. campestris*-like mosquito should be carried out as they were major proteins in the female salivary glands.

In this study, SN 5, 7, and 18 were found only in the medial lobe with unknown function whereas SN16 and 17 proteins were expressed only in the distal-lateral lobes with no significant match with protein sequences in the database. These proteins should be identified and characterized as they may be involved in blood feeding and/or pathogen transmission.

In conclusion, the proteins in the salivary glands of male and female *An. campestris*-like were analyzed for the first time using a proteomic approach. SDS-PAGE analysis revealed at least 12 major proteins. Two-dimensional gel electrophoresis showed approximately 20 major and several minor protein spots. Also, at least 15 glycoproteins were detected in the female glands. The different morphological regions of the female salivary glands displayed distinct electrophoretic protein profiles. Blood feeding proteins, i.e., putative 5'-nucleotidase/apyrase, anti-platelet protein, long form D7 salivary protein, D7-related 1 protein, and gSG6, were detected in the distal-lateral lobes and/or medial lobes of the female glands. Similar electrophoretic protein profiles were detected comparing the male and proximal-lateral lobes of the female glands, suggesting that these lobes are responsible for sugar feeding. Proteins identified by this approach

could be tested in strategies developed to control pathogen and disease transmission and studied regarding their roles in hematophagy.

Unpublished data: Proteomic profiles of female salivary glands distinguish species in *Anopheles barbirostris* complex (Diptera: Culicidae)

Proteomic profiles of female salivary glands of the five sibling species were compared and analyzed using 2-DE and nanoLC-MS to search for variation of proteins in the glands (Fig. 1). However, less than half of numbers of the major proteins of each species were identified. Four protein families were commonly found in the salivary glands of the five sibling species including apyrase/5'-nucleotidase, anti-platelet (GE-rich/30 kDa), D7/D7-related, and gSG6. Identification of the major proteins by Denovo sequencing or other proteomic methods should be performed in the future. These results could lead to further investigation of the similarities and differences in salivary components among the five species on their evolution of blood feeding, host seeking and pathogen the ransmission and might be useful for construction of an additional tool to distinguish the sibling species.

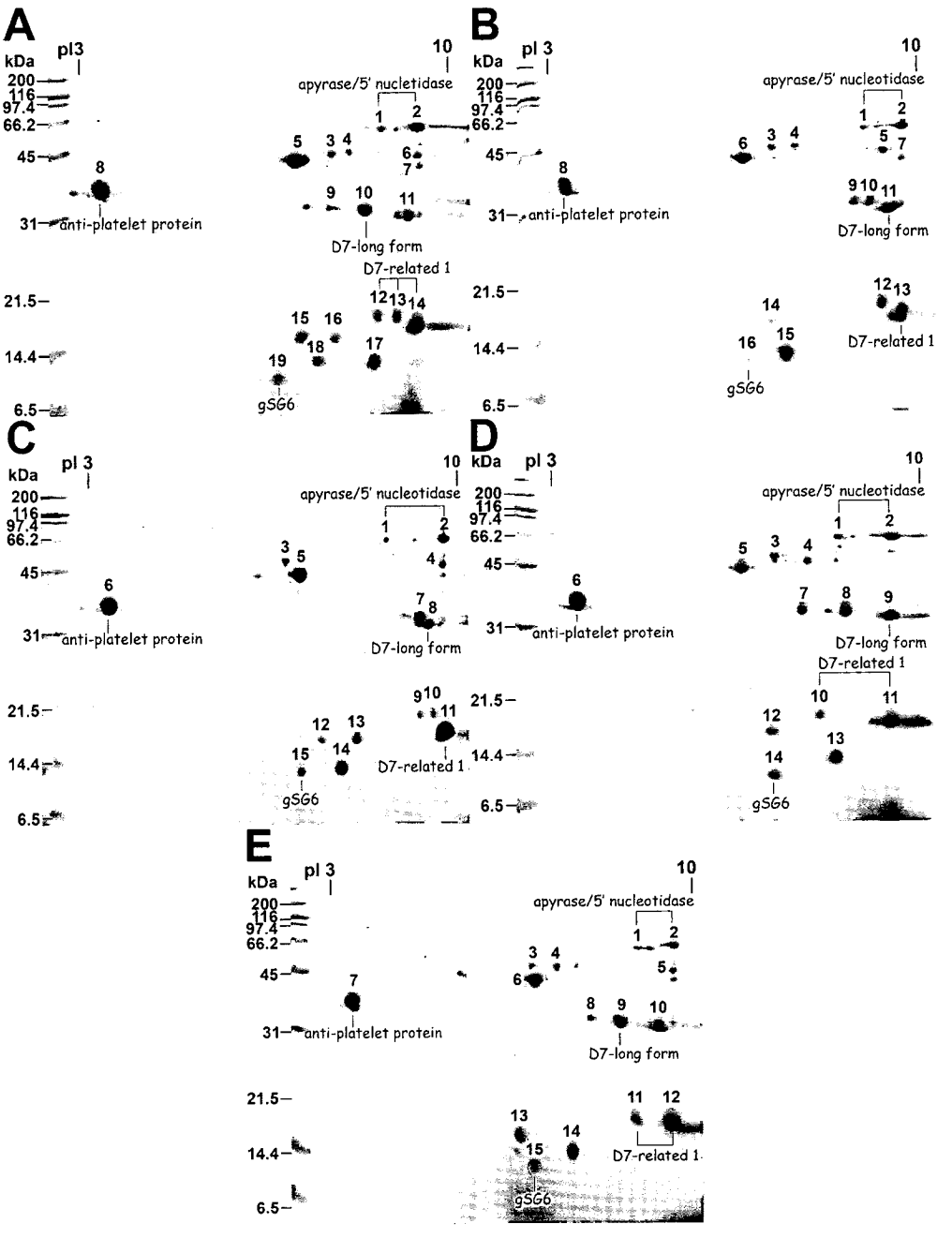

Fig. 1 2-DE analysis of female salivary glands of *An. barbirostris* complex. Proteins were separated on Immobiline DryStrips 7 cm, pH 3-10. Separation in the second dimension was performed using 15% SDS-PAGE. The gels were stained with Coomassie blue. Numbers indicate major salivary gland proteins. Molecular mass markers are indicated on the left in kDa. Isoelectric points (pI) are indicated at the top. A: a representative of 2D gels of 35 whole female salivary gland pairs of *An. campestris*-like; B: *An. barbirostris* species A1; C: *An. barbirostris* species A2; D: *An. barbirostris* species A3; E: *An. barbirostris* species A4.